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Immobilized Jagged1 for Notch3-specific Differentiation and Phenotype Control of Vascular Smooth Muscle Cells

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Abstract

Notch signaling plays a critical role in regulating vascular morphogenesis. In vascular interventions, the endothelial cells (ECs) are often damaged, and EC-SMC contact is compromised. The objective of this study was to investigate if immobilized Jagged1 can act as an EC-surrogate material to direct and control vascular smooth muscle cell (VSMC) behavior via Notch signaling. It was shown that immobilized Jagged1 induced vascular differentiation of iPSC-derived mesenchymal stem cells and mouse embryonic multipotent cells. Immobilized Jagged1 was insufficient to induce mature contractile markers in coronary artery SMCs; therefore, serum starvation and TGFβ1 treatment were investigated. Although Notch signaling is mechanosensitive in nature, it was also determined that a pulling force was not needed for Notch3 activation. Overall, it is concluded that immobilized Jagged1 is an essential regulator in SMC phenotype and SMC differentiation. These findings may have clinical relevance for modulating VSMC phenotype in cardiovascular disease states and in tissue engineering.

Keywords: Notch3 Signaling, Jagged1, Vascular Smooth Muscle Cells, Phenotype Regulation, Mechanotransduction

Summary for Lay Audience

To treat atherosclerotic vessels, stent deployment is a common intervention, but unintentional damage to the endothelial cell (EC) layer can cause smooth muscle cell (SMC) dysregulation. The Notch signaling pathway plays a critical role in regulating SMC phenotype switching through Jagged1-Notch3 signaling between EC and SMCs. Little is known on biomaterial approaches to direct Notch signaling and how ligand presentation strategies affect SMC response. Therefore, this study proposed bead-bound Jagged1 cell surrogates as a model in regulating the contractile VSMC phenotype. This study aimed to determine how immobilization strategies, crosstalk and cell source affected signaling response. Jagged1 was attached to magnetic nanoparticles and targeted binding to the Notch3 receptor on human coronary artery SMCs, iPSC-MSC, or pre-differentiated 10T1/2 cells. The use of bead-bound Jagged1 suggests high potential in modulating the development and maturation of the vasculature. Findings may have clinical importance and therapeutic potential for modulating vascular SMC phenotype during various cardiovascular disease states and in tissue engineering, with the possible application for bioactive stent materials.

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Thank you.

Kathleen Zohorsky

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List of Abbreviations

Cell Types

BMSCs	Bone marrow-derived mesenchymal stromal cells
EC,	Endothelial cell
iPSC-MSC	Induced pluripotent stem cell-derived mesenchymal stem cells
MSCs	Mesenchymal stem cells
SHEDs	Human exfoliated deciduous teeth stem cells
SMC	Smooth muscle cells
10T1/2	Mouse embryonic multipotent mesenchymal progenitor cells

Signaling Proteins and Growth Factors

BMP	Bone morphogenetic protein
EGF	Epidermal growth factor
MAPK	Mitogen-activated protein kinase
Shh	Sonic hedgehog
PDGF B	Platelet-derived growth factor B
TGFβ	Transforming growth factor β
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site
YAP/TAZ	Yes-associated protein/PDZ-binding motif,

Notch Components

DLL	Delta-like ligand
DSL	Delta/Serrate/Lag2 family
HD	Heterodimerization domain
HES	Hairy enhancer of split
HEY	HES related with YRPW motive
Jag	Jagged
LNR	Lin12/ Notch related domain
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain
TM	Transmembrane domain

Gene/Protein Nomenclature

Acta2	Smooth Muscle α-Actin
Cnn1	Calponin
Myh11	Smooth muscle myosin heavy chain
Smtn	Smoothelin

Other Abbreviations

BAS	Bioactive stents
CAD	Coronary artery disease
CVD	Cardiovascular disease
DES	Drug-eluting stents
DMEM	Dulbecco's modified eagles' medium
ECM	Extracellular matrix
Fc	Fragment crystallizable region
FN	Fibronectin
GSI	Gamma-secretase inhibitors
HBSS	Hanks balanced salt solution
mAbs	Monoclonal antibodies
MI	Myocardial infarction
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
pN	Piconewton
TGT	Tension gauge tethers

Chapter 1. Introduction

This chapter provides an overall introduction and objectives of the thesis work.

1.1. Overview

The incidence of cardiovascular diseases (CVD) is one of the major causes of death worldwide and is a leading concern to medical professionals worldwide. Over 2 million Canadians live with diagnosed ischemic heart disease, and over 150 000 Canadians are newly diagnosed a year, indicating the high prevalence of heart disease in Canada[1]. Atherosclerosis is one of the leading causes of coronary artery disease (CAD) pathogenesis and is a major underlying cause of CVD. Atherosclerosis is the build-up of fatty plaque-like material in the intima of coronary, carotid and peripheral arteries causing narrowing and reduced blood flow[2]. While angioplasty is a common intervention for mild blockages, for severe CAD (e.g., multiple blockages of coronaries bypass, grafting is needed (which include autologous vessels or synthetic grafts).

Coronary artery bypass grafts are very invasive and have complications including thrombosis and neointimal formation, leading to graft failure, donor site morbidly, and immune rejection. The emergence of tissue-engineered blood vessels to overcome these drawbacks aim to produce functional substitutes and are being extensively investigated[3–5]. An alternative is to take a less invasive surgical approach by deploying stents through balloon angioplasty surgery to restore blood flow in the vessel. Coronary artery stents, including metal stents, drug-eluting stents (DES), biodegradable stents, and bioactive stents (BAS) have been designed [6–8]. These designs have shown some success; however, restenosis is still a major issue that has been attributed to vascular arterial damage of the inner endothelial cell layer, suspected to occur during stent deployment[9,10].

There is a direct dependence that the smooth muscle cell (SMC) phenotype has on the presence of endothelial cells (ECs)[11–15], and there have been mechanisms proposed that govern their cellular interactions. Notch signaling has been suggested as a major regulator in the vasculature, and the Notch driven relationship helps to control SMC phenotype regulation[13,16]. Many researchers have investigated factors that control SMCs, including; matrix components, growth factors, scaffold geometry, mechanical stimulation, as well as coculture interactions[14,17]. Although it is hard to fully recapitulate the native EC-SMC interactions, there is a push towards trying to imitate native function and trigger a proper cellular response. Many bioactive/biomimetic stents have been proposed to mimic natural cell-cell communication. The Notch ligand Jagged1 could be a potential bioactive protein to maintain SMC contractile function and can be incorporated into stents or other biomaterial design. Understanding the mechanism from a biological perspective and translating this knowledge into tissue engineering can allow the design of more accurate and physiologically relevant stents or biomaterial substitutes. These can be incorporated to improve the signaling capability of Notch signaling. Therefore, a deeper understanding of biomaterial-directed Jagged1 Notch signaling behavior in the context of controlling SMCs in the vasculature will enable functional the design of functional engineered substitutes for the treatment of vascular diseases.

1.2. Thesis outline

This thesis has five chapters that outline and detail the work carried out. Chapter 2 introduces the role of Notch signaling in developmental biology, the role of Notch signaling in the vasculature, as well as current proposed strategies to modulate and control Notch signaling in a tissue engineering context. Furthermore, it illustrates the motivation for this study; to develop a functional stent material to improve stent restenosis and outlines the thesis objectives. The

materials and experimental methodologies used are described in Chapter 3 followed by a discussion on significant research findings of this work in Chapter 4. Finally, Chapter 5 summarizes the research and provides the significance and future directions.

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Chapter 2. Literature Review *

This chapter discusses the relevant background information and current progress in achieving Notch signaling biomaterials for vascular tissue engineering and regenerative medicine approaches.

*A portion of this chapter (**Section 2.4** and following) was expanded and published in a recent review paper:

Kathleen Zohorsky and Kibret Mequanint. Designing biomaterials to modulate Notch signaling in tissue engineering and regenerative medicine. *Tissue Eng. (B).* ***2020*** doi.org/10.1089/ten.TEB.2020.0182

2.1.Structure and function of blood vessels

Blood vessels are a part of a complex network that makes up the vasculature and allow blood, oxygen, and nutrient transfer throughout the body. Oxygen-rich blood leaves the heart through the aorta, which branches into arteries, smaller arteries, arterioles, and finally capillaries. Capillaries are permeable to oxygen (moves from the capillary to the cells) and carbon dioxide (moves from the cells to the capillaries). The oxygen-depleted blood is then transported back through the capillaries, into small venules, veins, and finally enters the vena cava into the upper right atrium of the heart. Apart from the small arterioles and capillaries, the functional blood vessels are composed of three distinct concentric layers that vary in function depending on the location and purpose of the vessel. The structure and development of arteries and veins both differ due to the various functions that they play within the body. Veins have a functionally thinner tunica intima than that of arteries because they do not have a primary contractile function. Arteries have a much thicker tunica intima. The structure of a muscular vascular wall (eg. Coronary artery) and its cellular components are shown in **Figure 2-1**.

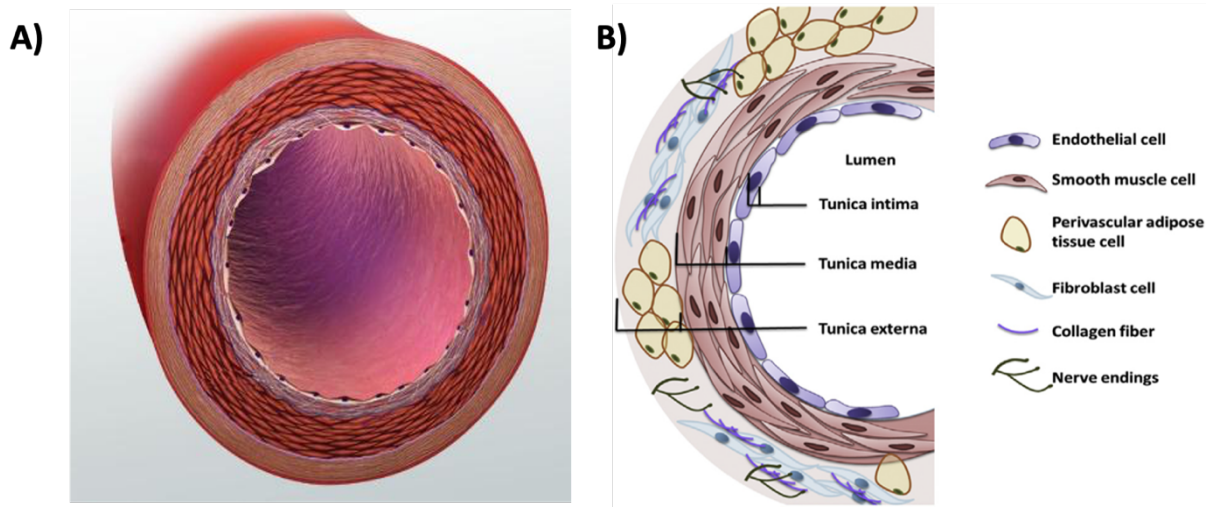

Figure 2-1 Structure of a muscular vascular wall (e.g., coronary artery)
A) Artery cross-section **B)** Structure of the arterial wall and the cellular makeup. Depiction of the three vascular layers, tunica intima, tunica media, and tunica externa which come together to form the vascular wall (Reproduced from ref [1] with permission Copyright © 2015 Zhao, Vanhoutte, and Leung, Production and hosting by Elsevier B.V).

2.2.Components of the vascular wall

The vascular wall is made up of three layers; tunica intima, tunica media, and the tunica externa. The innermost layer of the artery which lines the lumen of the vessel is the tunica intima (also referred to as the endothelium) which consists of a monolayer of endothelial cells (ECs) attached to a basement membrane of extracellular matrix (ECM) proteins. The functional relevance of the endothelium is to protect and respond to external stimuli, which functions as a barrier between the blood flowing in the lumen and the surrounding tissues. The endothelium produces several vasodilator and vasoconstrictor substances that regulate not only vasomotor tone but also the recruitment and activity of inflammatory cells and control its tendency towards thrombosis[1–3]. By communicating with smooth muscle cells (SMCs) in the vascular wall, ECs can decrease or increase arterial diameter by altering its contraction and relaxation behavior[4].

Adjacent but in contact with the intima is the tunica media, which contains multiple layers of densely packed SMCs with fenestrated elastic lamellae interspersed between them. These SMCs

are capable of switching between a contractile phenotype found in physiological conditions and a synthetic phenotype, which is characteristic of developmental (proliferative), or pathological conditions[5]. There are many characteristic and functional differences between the two phenotype conditions. Cells in a contractile phenotype have a spindle-shaped morphology, a centrally located nucleus, and proliferate at a very low rate but have robust contractile protein. A morphology transition to a more synthetic phenotype mimicking that of fibroblasts occurs upon stimulation by various conditions such as inflammation, high levels of ECM production, mechanical forces, soluble factors, and signaling cues from ECs. The SMC phenotype continuum and modulation is presented in **Figure 2-2.**

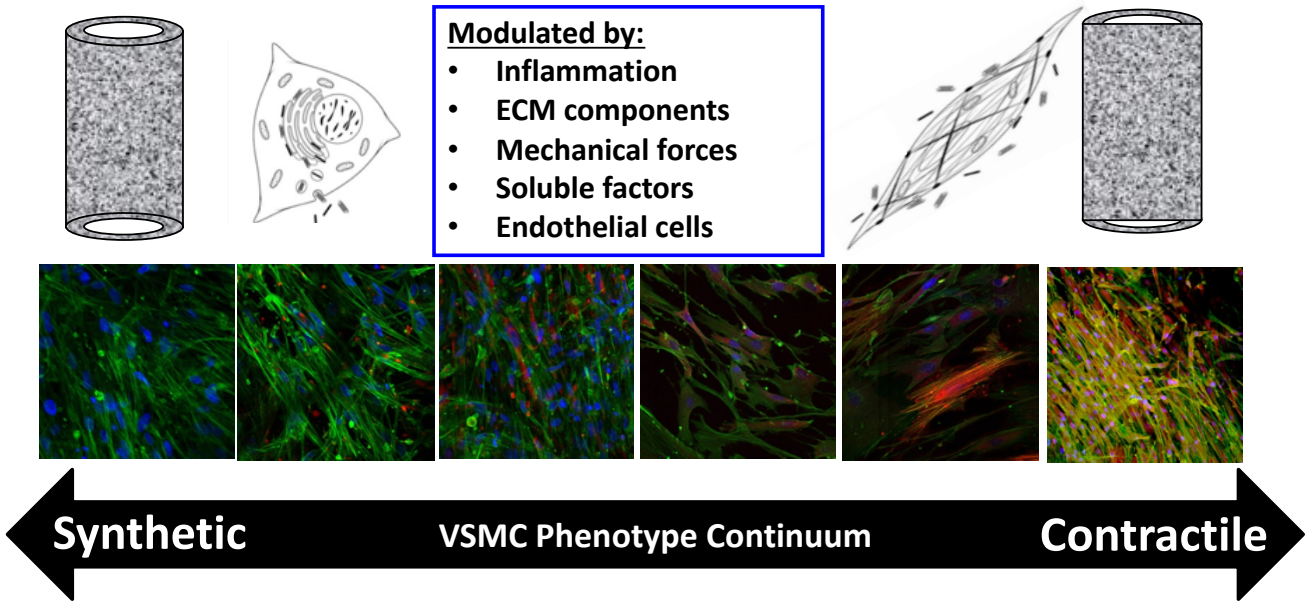

Figure 2-2. Phenotype regulation continuum of VSMCs.
Phenotype switching between a synthetic and a contractile phenotype is modulated by various biochemical and mechanical cues, including; inflammation, ECM components, mechanical forces, soluble factors, and endothelial juxtacrine signals. (confocal images legend; green: F-actin, red:Acta2; yellow: Cnn1. Images taken by Shigang Lin and Stephanie Grenier).

The key components of the contractile apparatus in VSMCs are smooth muscle myosin heavy chain (SMC-MYH) isoforms and SM α-actin (Acta2), along with the contractile filament smoothelin B which is expressed in mature VSMCs[5,6]. Other characterized markers of VSMC's

include transgelin and the calcium-binding protein and inhibitor of SM-MYH intrinsic ATPase activity, calponin[6]. Regulation of the actin cytoskeleton and contractile machinery of VSMCs are important for vascular homeostasis. Within their contractile phenotype, SMCs relax and contract in a cascade manner to increase or decrease the vessel diameter, thus changing blood flow rate through the lumen. Contractions can be modulated by mechanical (intraluminal pressure, lumen stretch/compression), electrical, or pharmacological activation[7].

The outermost layer of the artery is the tunica externa and is largely composed of fibroblasts and collagenous ECM proteins. This layer primarily functions to protect and anchor the artery to the surrounding tissue. In addition to these three aforementioned layers, the artery has internal and external elastic membranes. These elastic tissues aid the vessel wall to maintain structure and resilience and impart arterial elasticity under pulsatile flow. The mechanical integrity of the vessel is important to support external forces and maintain its intrinsic contractility. The mechanical properties of the blood vessel arise from a network of ECM components and their interactions with the cells. The matrix is composed of a network of fibrous proteins, mostly collagen and elastin, and a basement membrane composed of the elastic lamina, collagen IV, and fibronectin (FN) embedded in a hydrogel of proteoglycans and several glycoproteins[8]. These layers provide mechanical support, anchorage of the cells, guidance, the transmission of mechanical forces, and restricts the vessel from mechanical deformation[8].

Blood vessels experience various forms of mechanical forces; stretch (through muscle distention), cyclic strain (from pulsatile blood flow), compression (due to differential blood pressure), surface force (from systolic blood flow), and shear stress (blood drag force)[4,9]. These various forces control and regulate the function and homeostasis of the blood vessel. The

endothelial cells which line the lumen of the vessel can sense pressure and blood flow and they are capable of transducing changes in mechanical forces into changes of the SMC tone[3].

2.2.1. Structure and function of the coronary arteries

The coronary arteries originate at the root of the aorta and split into two branches that vascularize the myocardium. The left coronary artery supplies blood to the left atrium and left ventricle of the heart, while the right coronary artery supplies blood to the right atrium and right ventricle of the heart. Coronary arteries are very muscular by nature, having the bulk of the arterial wall made up of the media layer to provide this muscular tone. The elastic laminae surrounding the tunica media allow the artery to recoil and prevent vascular dilation that would result from the creep of collagen under high blood pressures[10]. Properties of the coronary artery including diameter, stress, wall thickness, transport blood pressure, burst pressure, and longitudinal strains can be summarized in **Table 2-1.**

Table 2-1 Properties of the coronary artery

Property	Reported Range [8]
Internal diameter	~ 3- 4 mm
Stresses	0.75 -2.25 Pa
Wall thickness	~ 1 mm
Transport blood pressure	80-120 mmHg
Burst pressure	~ 2000 mmHg
Longitudinal strains	10-15 %

2.3. Diseases of the coronary artery

Since coronary arteries deliver blood to the heart muscle, all coronary artery disorders/diseases can have serious health implications. Cardiovascular disease (CVD) is defined as any physiological condition that causes the impaired function of the heart or blood vessels within the circulatory system. Myocardial infarction (MI) is often the consequence of prolonged CVD and results due to the blockage of blood supply to the myocardium caused by ischemia (lack of oxygen and nutrients) in one or more coronary arteries[11]. Coronary artery disease (CAD) is a type of CVD and is one of the leading causes of morbidity and mortality in the world[12]. The development of atherosclerotic lesions in the coronary artery is one of the central components of CAD. Atherosclerosis is characterized by the buildup of plaque in the inner lining of an artery causing it to narrow or become blocked and is the most common cause of heart disease and is a leading cause of CVD and MI[13]. Major risk factors for atherosclerosis are aging, diet, diabetes, smoking, lifestyle, and genetics. The three stages of development in atherosclerotic lesions are depicted in **Figure 2-3**. A depiction of the normal muscular vascular in which the vessel is in homeostasis (**Fig. 2-3 A)** can be compared and contrasted to the development and progression of atherosclerosis (**Fig. 2-3 B-D)**.

Atherosclerosis can be caused by many biological mechanisms, including endothelial dysfunction, which creates increased permeability of the endothelial barrier and allows for the abnormal accumulation of plasma-derived lipids and oxidation products within the arterial wall[14]. The retention of low-density lipoproteins' creates a vascular response inducing leukocyte adhesion, the recruitment of inflammatory cells to the injury site, and recruitment of monocytes from the peripheral blood, which become activated macrophages[13,15].

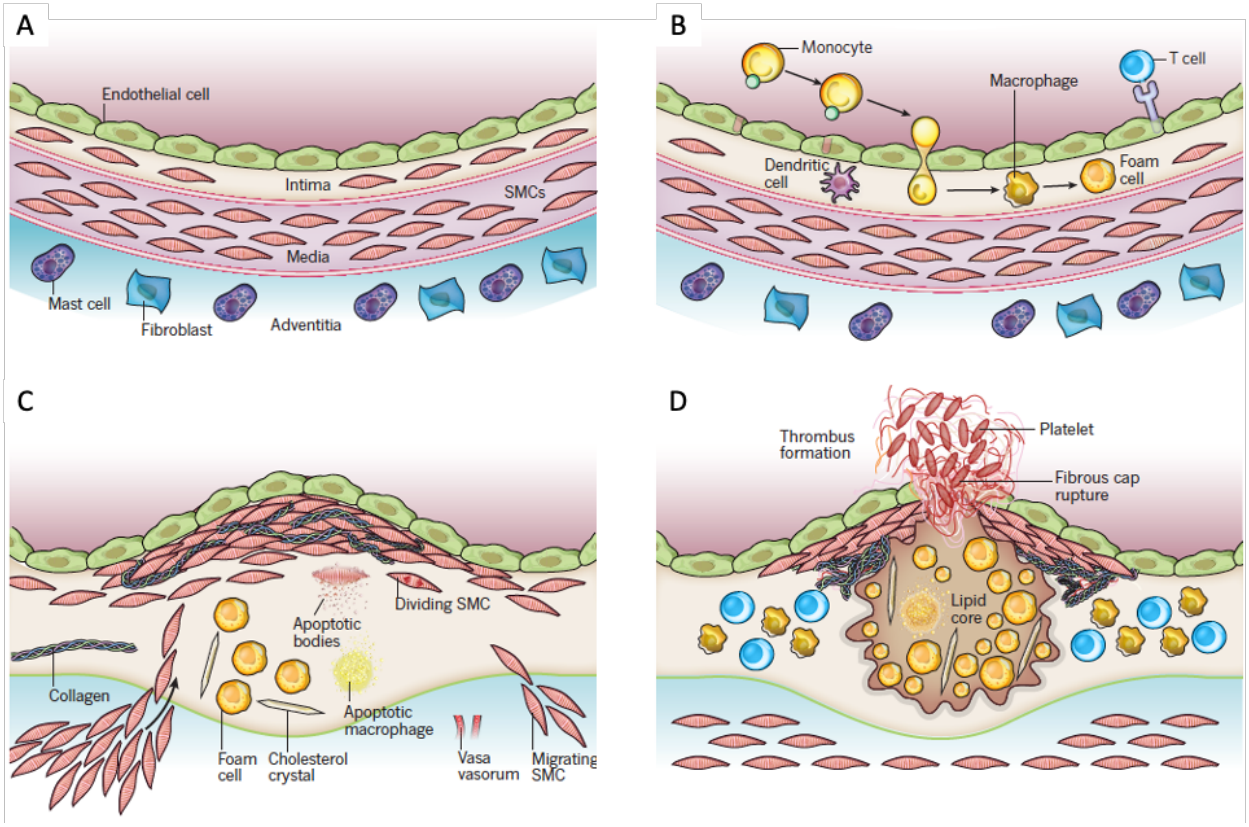

Figure 2-3 Stages of development of atherosclerotic lesions
A) The 3 normal artery layers (intima, media, and adventitia), and overall vessel homeostasis. **B) Stage1**-Adhesion of blood leukocytes to the activated endothelial monolayer, migration of leukocytes into the intima, maturation of monocytes into macrophages and uptake of lipids creating foam cells. **C) Stage 2-** lesion progression involving migration of SMCs from the media into the intima, proliferation of SMCs and heightened synthesis of extracellular matrix macromolecules. Cell death and apoptosis of both macrophages and SMCs are often seen in advancing plaques, and the extracellular lipids accumulate in the central region of the plaque, creating the lip or necrotic core. **D) Stage 3** – Thrombosis and ultimate complication, including physical disruption of the plaque. Depicted here is the rupture of the plaques fibrous cap and thrombus formation. (Reproduced with permission from ref [15]. © Copyright 2011 Macmillan Publishers Limited.)

The altered cell state and lost cell communication is one aspect that contributes to the development of fatty plaque deposits[14]. The dysregulation of the artery wall also leads to SMC phenotype switch and subsequent migration of the SMCs from the medial layer into the intima where they synthesize matrix molecules that mature the plaque further. Atheroma formation includes components like foam cells, lipid-laden macrophages, and SMCs from the tunica media. The SMCs also produce

ECM molecules, including interstitial collagen and elastin which form a fibrous cap that covers the plaque[14]. This multistep response by the body ultimately aims to protect and strengthen the weakened vessel wall, however inadvertently, the artery lumen narrows and causes serious health complications. Recently an in-depth analysis of atherosclerosis biology and the progress and challenges of biological translation and treatment has been reviewed [15]. This is important to gauge the understanding of various treatment options for cardiovascular diseases and atherosclerosis.

2.3.1. Atherosclerosis treatment options

Both surgical and pharmacological methods are used for atherosclerosis treatment. The most common surgical techniques used to treat cardiovascular diseases are i) coronary artery bypass surgery and ii) balloon angioplasty and stenting. Coronary artery bypass grafting is an invasive surgical method. These grafts are primarily sourced from autologous tissues[16,17], however other allograft and xenograft tissues are available (but these are associated with a high risk of immune rejection and potential disease transmission). Patients with unavailable autologous tissues can also receive synthetic grafts such as Teflon® and Dacron®. Research into developing synthetic tissue-engineered vascular grafts is still ongoing to improve the design and minimize complications.

Coronary artery angioplasty is another common, less invasive surgical technique used to restore blood flow in an affected artery. Stents are inserted through a thin tube (catheter) into the narrowed part of an artery. A wire with a deflated balloon is passed through the catheter and the balloon is then inflated, compressing the fatty deposits against the artery walls. Stents are often left in the artery to keep the artery open, and some stents slowly release medication to help keep the arteries open. Stent technologies can be grouped into six categories (**Appendix A1: Advantages and disadvantages of current stent technology)**: a) bare b) coated metallic stents, c) drug-eluting stents (DES) e) biodegradable stents and f) bioactive stents (BAS). The

development of stents was driven because of acute vessel closure[18]; however, one major clinical concern with all stent designs is in-stent restenosis[19,20]. Stent design has progressed, but there is no perfect solution to stop this complication.

2.3.2. Stent-induced the coronary artery injury

In-stent restenosis, defined as the gradual re-narrowing of a stented coronary artery lesion due to arterial damage with subsequent neointimal tissue proliferation, is a major clinical problem. Restenosis has shown to occur following angioplasty in 20-40% of coronary lesions within a 6-month postoperative timeframe[18]. Restenosis can be attributed to many types of vascular injury, including EC dysfunction as well as ECM disruption. The deployment of the stent can cause mechanical damage (splitting atheromatous plaque and stretching of the vessel wall) to the arterial wall or scraping of the endothelial layer and inadvertently damaging endothelial cells[21,22]. Injury mechanisms of the vasculature have been examined *in vivo*, *in vitro*, and have been computationally modelled[23–26]. Overall, it has been proven that the integrity of the EC monolayer is important in maintaining vascular homeostasis[3,27,28]. The EC-SMC communication loss can cause VSMCs to undergo a phenotype switch from a contractile to a more proliferative synthetic type. It is this synthetic phenotype that have a high proliferative capability and are observed to migrate into the lumen and cause restenosis of the artery. Additional to signals from ECs, the disruption to the surrounding ECM has also been shown to affect changes in SMC phenotype and activation; matrix-degrading metalloproteinases degrade the collagen in the ECM, which are known to regulate VSMC behavior[29].

Although stent technology has made considerable progress in terms of improving patient outcomes, no single technology on the market has been completely successful. Thus, using biological mechanisms occurring naturally in the arterial wall allows us to create innovative

engineered solutions. If biological factors are incorporated into stents or biomaterials, it will enhance treatment outcomes for vascular disorder and disease progression, which could provide useful insights when exploring novel bioactive stents and bioactive materials.

2.4. Cell signaling pathways

Studying native cell signaling pathways and their influence on SMC response becomes increasingly important to provide insight on better designs for tissue-engineered solutions. Within the vasculature, cells continuously send and receive signals that are essential for development, homeostasis, and repair of various tissues and organs. They are able to adjust to their microenvironment and communicate with each other through a complex network of signaling pathways. In general, in the body, cells can receive information through 4 types of signaling depending on the tissue type and the distance the signal has to travel; autocrine, endocrine, paracrine, and juxtacrine (**Fig. 2-4 A-D**). In tissue engineering, cells signal predominantly by paracrine or juxtacrine signaling[30]. The difference between paracrine and juxtacrine signaling is the mode of ligand presentation[31,32]. In paracrine signaling, the ligand is secreted by one type of cell and is released into the neighboring target cells as a diffusible soluble factor. In juxtacrine signaling, the ligand is anchored on the signal-sending cell surface to bind the receptor on another cell, thus requiring cell-cell contact for proper function.

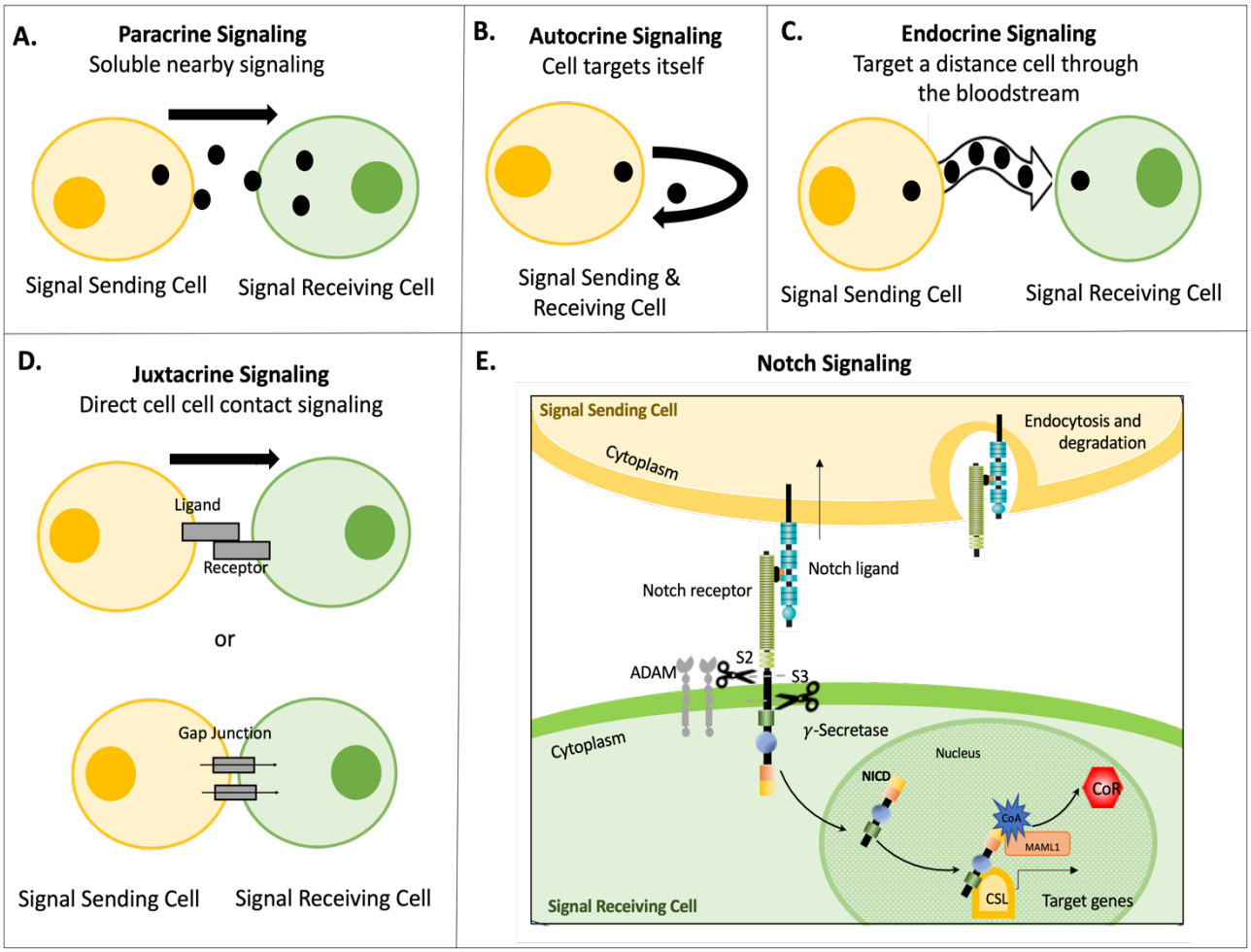

Figure 2-4 Cell signaling types and an overview of the canonical Notch signaling cascade. **A)** Paracrine signaling is short-range signaling using soluble factors. **B)** Autocrine signaling signals itself using various excreted factors. **C)** Paracrine signaling is a long-range signaling type delivered through the bloodstream. **D)** Juxtacrine signaling is a short-range signaling where cell-cell contact is needed through ligand-receptor interactions or gap junctions. **E)** The Notch signaling pathway a common juxtracrine signing pathway and plays an important role in the vasculature. Published as Kathleen Zohorsky and Kibret Mequanint. Tissue Eng. Part B, © Copyright 2020, Mary Ann Liebert, Inc.

Common intracellular signaling mechanisms in the body include the Wnt signaling, the sonic hedgehog (Shh) signaling, the bone morphogenetic protein/transforming growth factor β (BMP/TGFβ) signaling, and Notch signaling, to name a few. Notch signaling is an important heterotypic and homotypic juxtacrine cell-to-cell signaling pathway that is active in numerous cell

fate decisions, including development, tissue maintenance, homeostasis, as well as disease progression[33–35]. Its various tissue-specific roles allow for Notch signaling to be a promising target for cellular control. Most importantly, Notch signaling plays a vital role in the vascular. Notch signaling in combination with other pathways also allows for signaling crosstalk which can be therapeutically useful[36–38]. In view of this, Notch signaling, and Notch modulation will be discussed further.

2.5. The Notch signaling pathway

Notch signaling is simple in design with few core signaling components, as seen in **Figure 2-5,** however it is complex from a regulation perspective as it affects numerous distinct cell fate decisions and is important in the development of many tissues[39]. Notch signaling is a critical heterotypic juxtacrine cell-to-cell signaling pathway that is active in numerous cell fate decisions, including development, tissue maintenance, homeostasis, as well as disease progression[33–35]. Its various tissue-specific roles allow for Notch signaling to be a promising target for cellular control.

Notch signaling is an evolutionarily conserved mechanism regulated by interactions with transmembrane proteins of the Jagged or Delta-like (Dll) family of ligands. Mammalian tissues express various combinations of four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five Notch ligands (Jagged1, Jagged2, Dll1, Dll3, Dll4) and signaling is activated through three sequential cleavages, named as S1, S2, and S3 cleavages. In S1, Notch is cleaved into a heterodimer by Furin-like convertase, which undergoes O-fucosylation by O-Fucosyltransferase and glycosylated by Fringe in the Golgi before the receptor is transported to the cell membrane. This processing step controls the abundance of Notch receptors at the cell surface. The binding interaction between a specific Notch receptor with a corresponding ligand initiates the regulated intramembrane proteolysis, resulting in a conformational change of the Notch extracellular domain

(NECD) of the receptor. This conformational change exposes an S2 cleavage site for the metalloprotease ADAM17/TACE to initiate. In the absence of ligand binding, the Notch receptor is maintained in an autoinhibited and protease-resistant state[40]. There is extensive evidence implicating NECD endocytosis in Notch signaling force generation (this is expanded in **Section 2.7.2**); however, the mechanism and purpose is still highly debated [40–44].

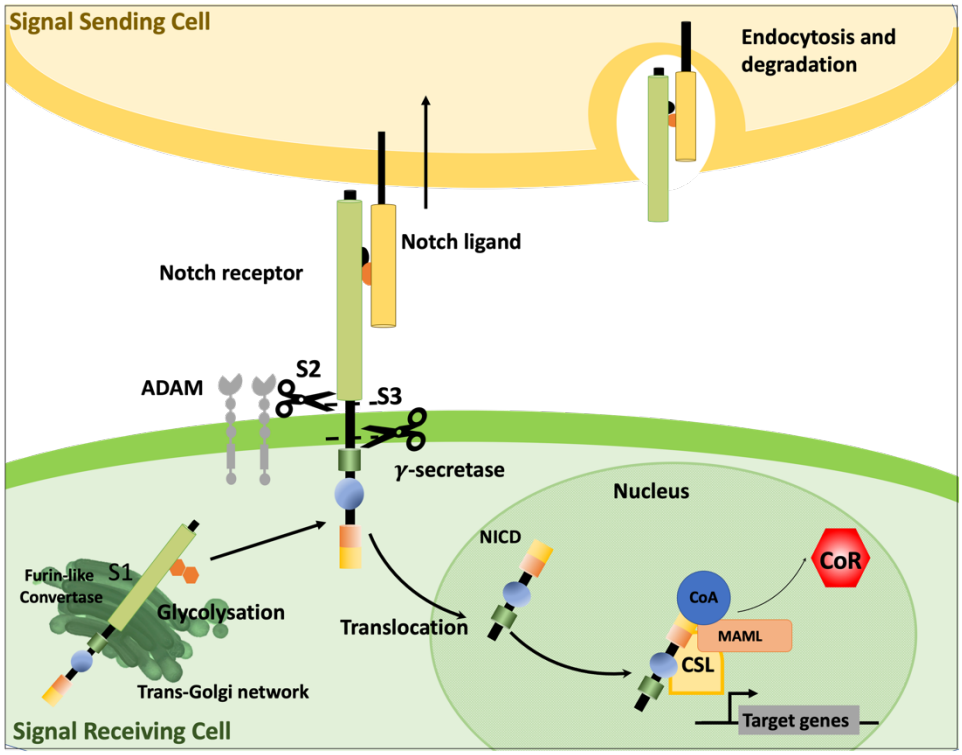

Figure 2-5 The Notch signaling cascade.
Detailed description of this pathway provided in the text. Published as: Kathleen Zohorsky and Kibret Mequanint. Tissue Eng. Part B. 2020 , © Copyright 2020, Mary Ann Liebert, Inc.

Full activation of Notch is achieved upon S3 cleavage of the Notch extracellular truncation (NEXT) by presenilin, the proteolytic subunit of the γ-secretase complex, which cleaves the Notch intracellular domain (NICD). Once released from the plasma membrane, the NICD translocates into the nucleus, where it binds to the transcriptional repressor RBPJ (recombination signal-

binding protein for immunoglobulin κ J region, also known as CSL and CBF1). Finally, the NICD activator complex promotes transcription of downstream gene targets, including Hairy Enhancer of Split (*HES*) and HES related with YRPW motive (*HEY*), as well as various downstream functional genes and proteins[45,46]

Unlike other signaling pathways that are enzymatically amplified, Notch signaling instead depends on stoichiometric receptor-ligand interactions to activate it, and any imbalance may inhibit the process[39,47]. From what seems like a simple mechanism when compared to other signaling pathways, the regulation and modulation of Notch signaling is very complex and tissue specific. Distribution of the Notch signaling components in different tissues vary considerably, thus interpreting these context-specific effects of Notch will ultimately require that the wiring of the regulatory networks in which it operates is understood[34]. The importance of specific ligand-receptor pairings to develop new therapeutic targets must be investigated. Understanding the role of Notch in tissue specification during development and disease state is beneficial to translate these mechanisms into tissue engineering[48]. Although the principle of tissue engineering can be applied to many tissues, the focus of this research is on vascular tissues. In view of this, important biological processes in the vasculature driven by Notch signaling will be identified.

2.5.1. Notch modulation in the vasculature

Vasculogenesis is the differentiation of precursor cells (angioblasts) into ECs and the *de novo* formation of a primitive vascular network, whereas angiogenesis refers to the growth of new capillaries from pre-existing blood vessels. Notch signaling is critical in both vasculogenesis during development and in angiogenesis[49,50]. It protects the endothelium[51], controls VSMC phenotype[52–55], promotes neoangiogenesis[56–58], tip-stalk cell patterning[59], and regulates arteriovenous specification[60,61]. The various roles of Notch signaling in the vasculature, including

its influence on ECs and SMCs have been reviewed previously[35,62]. In this section, important Notch receptor-ligand pairings in the vasculature are highlighted. VSMCs dominantly express Notch1, Notch2, and Notch3 receptors, while ECs express Notch ligands Jagged1, Jagged2, Dll4, and to some extent Dll1 in the remodeling vasculature[6,63]. The context in which these ligands and receptors occur is essential; however, the anatomical location within the vascular bed and the associated physiological forces (flow and stress) are likely to impact the context-dependent Notch activation[56].

The growth of the vascular system involves tip cell selection, sprout formation, tip cell migration, stalk cell proliferation, and ultimately vascular stabilization[64] which are collectively influenced by Notch signaling. Vascular sprouting (**Fig. 2-6 A**) is guided by the migration of tip cells in response to a matrix-bound vascular endothelial growth factor (VEGF) gradient[59,65], with Dll4 acting downstream as a negative regulator[66,67]. Tip-stalk cell fate plays a significant role in angiogenesis since tip cells direct new blood vessel growth. Interestingly the role of Notch in both tip and stalk cells is evident within the distribution patterns of Notch signaling ligands and receptors. VEGF signaling induces Dll4 in tip cells, tip cells then suppress tip-cell features in adjacent stalk cells via Dll4/Notch-mediated lateral inhibition[59]. Simultaneously Jagged1 antagonizes Dll4-mediated Notch activation in stalk cells to increase tip cell number which consequently enhances vessel sprouting[68]. Hence it is this Jagged1 and Dll4 "salt-and-pepper" pattern that dictates the tip-stalk cell phenotypes within this niche. The function of Notch signaling varies greatly depending on the location of the vascular bed and also the type of the vessel. The arteriovenous specification is established early in development through a variety of transcription factors. A key Notch-defining factor in this specification is the Notch3 receptor found in arterial SMCs, which is notably absent in veins, and Jagged1 expression by ECs are responsible for this

maturation. The transcription factors Foxc1 and Foxc2 and VEGF signaling are primarily responsible for arterial fate[61,69]; the upregulation of these transcription factors results in increased expression of Dll4. In contrast, vein identity is regulated by the repression of Notch1[61].

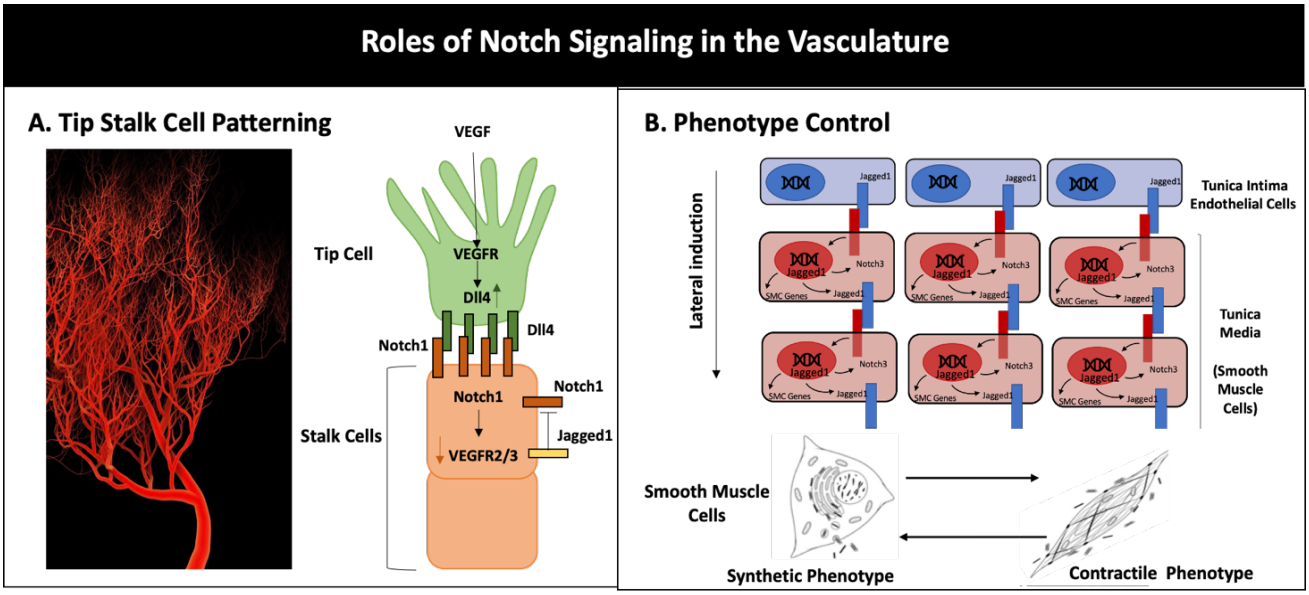

Figure 2-6 Roles of Notch signaling in the vasculature.
Some major roles in the vasculature, including **A)** Tip stalk cell patterning utilizing Dll4, Jagged1 signaling, and **B)** Phenotype control of vascular smooth muscle cells through Jagged1 lateral induction.

Recruitment of mural cells (VSMC and pericytes) and the formation of a fully endothelialized lumen are hallmarks of arterial vessel maturation during development[61]. Upon activation of Notch in VSMC, ligand-receptor signaling is initiated throughout the VSMC lamellae by the process of lateral induction (**Fig. 2-6 B**). The propagation of Notch signaling is crucial for regulating VSMC phenotype throughout the vascular wall, and hence is a critical phenomenon for inducing differentiation of the complete VSMC layer toward the homeostatic contractile phenotype[70]. Notch3 targeting is more prominent for SMC control and differentiation[52,71,72]. Notch1, in contrast, has been shown to regulate EC metabolism, proliferation, and monolayer regeneration[73]. Communication between ECs and SMCs in the vascular wall is essential, thus

diminished Jagged1 expression in ECs leads to abnormal smooth muscle development. Endothelial expression of Jagged1 is required for activation of Notch3 on VSMC maturation, differentiation, and contraction[74].

The impact of physiological forces within the vascular system on Notch signaling becomes apparent when considering the ability of the endothelium to respond to force patterning. ECs modulate their Notch component expression in response to hemodynamic forces. For instance, Notch1 activation in EC is sensitive to blood flow, where high shear stress has a critical role in the acquisition and maintenance of arterial indentation via its role in endothelial quiescence[75]. The indication that EC appears to respond to predetermined arterial or venous patterns is evident through current research, and there is a certain level of plasticity cues of the local niche suggested to be imposed by physical forces, such as hemodynamics[76]. Taken together, ligand specificity and selective Notch activation regulate differential phenotypes and function within the vascular tissue[70,77,78]. Understanding the tissue distribution of Notch signaling components is highly relevant to translate them into tissue engineering and regenerative medicine strategies.

Both ECs and SMCs have been implicated in arterial injury and disease states linked to dysregulation of various signaling pathways, including the Notch signaling pathway. It has been shown that upon arterial injury in the SMCs, Notch receptors and ligands as well as Notch transcription factors (HERP and HES) were coordinately downregulated after arterial injury[79]. Loss of function of these various ligands and receptors caused by trauma can create distortions in the microenvironment, where specific cellular fates can be dysregulated. The communication between SMCs and ECs within the vascular wall is important to control the proper function of the vasculature. Events such as disruption of the endothelial monolayer, growth factor exposure, interactions with the extracellular matrix, injury/wound healing, and vascular remodeling can

cause cascades in VSMC response leading to a rapid downregulation of contractile proteins and developing a highly proliferative and migratory phenotype[6].

Specific to this research, Atherosclerosis has been directly linked to Notch signaling dysregulation. Atherosclerotic lesions have been speculated to develop in arteries characterized by disturbed blood flow and low shear stress[15,80,81]. Lower levels of Notch signaling components were found in atheroprone regions, suggesting that disturbed blood flow could predispose those areas to atherosclerosis by affecting Notch signaling[74]. Additionally, characterizing gene expression profiles from plaque material in peripheral artery disease patients confirmed the role of Notch in atherosclerotic plaque stability, including stable or inflamed plaque gene expression profiles linked to Jagged1 and Dll4 expression in the plaque[74]. The phenotype switch from a contractile to proliferative/synthetic states (as discussed previously) is also a determinant for the development of atherosclerotic lesions[54]. Notch has been suggested to mediate activation and counteract trans-differentiation of SMCs as well as protect VSMCs from apoptosis, a major determinant of plaque instability[54,82]. Knowing this, Notch signaling has been proposed as a new therapeutic target in atherosclerosis[80], and the incorporation of native cell signaling in biomaterial design could prove valuable.

2.6.Notch signaling presentation strategies

An increasing need for regenerative medicine and tissue engineering approaches have been suggested to create functional cell-directing template biomaterials substitutes to recover lost function within a dysfunctional tissue[83]. Despite the multicellular nature of tissues, tissue engineering strategies often focus on seeding the main cellular component of a particular tissue to scaffolds and culture them for tissue maturation. Given the role of heterotypic Notch signaling in these tissues, the signal-sending cell (ligand-bearing cell) is often missing. Without endothelial

signals due to vascular injury, tissue engineering approaches have been proposed to provide the signal lost by ECs. In a biomaterial system, the strategy of presentation of Jagged1 can affect and direct cellular response greatly. One strategy is to present it as a soluble factor either by adding it together with culture media or using a delivery system. The soluble factor can be loaded to a biomaterial and released either by diffusion or by cleavage, delivering the soluble factor in a paracrine manner. Although the immobilization of paracrine signaling ligands to biomaterial surfaces is common, it is not required from a signaling point of view, it has been proved to slow the release profile and increase cellular accessibility to the delivered ligand/protein. The role of soluble Notch signaling is context-dependent and cell type-dependent.

Embedded and immobilized delivery of Notch ligands via a biomaterial surface facilitates juxtacrine cell-cell signaling found in the native tissues. Engineered biomaterials can be introduced to take on the role of the signal-sending cell and act as cell-surrogate biomaterials to replicate the cell-cell contact needed for signal propagation. Additionally, immobilized delivery allows for better spatial and temporal control. In general, signaling molecules can be presented to cells in one of three ways: i) adding them as a soluble factor, ii) direct conjugation or conjugation via a flexible molecular arm to a scaffold, iii) and affinity immobilization to the scaffold through antibody-binding proteins. The majority of research does allude to the need for ligands anchored or attached to a surface to effectively activate Notch signaling. The use of various soluble and immobilized factors can therefore influence Notch signaling and will be discussed.

2.6.1. Soluble factors to control Notch signaling

Various paracrine signaling factors including soluble Notch ligands, engineered decoy ligands[84], monoclonal antibodies (mAbs)[85,86], gamma-secretase inhibitors (GSIs)[87,88], and other soluble growth factors have been used to modulate Notch signaling. Engineered decoy ligands and

mAbs are potentially advantageous due to their specificity allowing for the targeting of individual Notch receptors. However, soluble Notch delivery and GSIs are more commonly used in treatment. Targeting via GSIs stop the release of Notch from the plasma membrane and the subsequent generation of the NICD. GSIs are intended to reduce the activity of Notch signaling and inhibit downstream effectors. GSIs can be delivered with or without a biomaterial carrier and can be used to therapeutically target and control ligand/receptor distribution and adverse cellular responses.

Likewise, soluble Notch ligands function through a paracrine manner (one cell secretes a ligand that can bind to nearby cells and induce a response). Inherently, bound and soluble Notch ligands compete for the available Notch receptors on the surface of a nearby cell. Soluble ligands can bind to Notch receptors but are, for the most part, unable to activate signaling; they rather appear to block signaling induced by trans-ligands in most cases[89]. Although soluble ligands have controversially activated Notch signaling, most commonly soluble Notch ligands have been applied to cell-based systems to competitively inhibit the Notch receptor active site. As it pertains to this thesis, the use of soluble Jagged1 to control cell behavior is reviewed in **Table 2-2.** As presented in this table, the influence of soluble Notch delivery is very context-dependent. Upon investigation, the use of soluble Jagged1 in the vascular seems to play an inhibitory role, inhibiting proliferation and neointima formation but should be explored further.

Table 2-2 Modulation of Notch signaling using soluble Jagged1

	Cell Type	Function	Role	Ref.
Multipotent Cells	Rat MSCs	MSC differentiation into cardiomyocytes	Notch Activation	90
	Peripheral blood mononuclear cells	Monocyte differentiation into M1, Mϕ with antimicrobial activity	Notch Activation	91
	Bone marrow-derived dendritic cells	Maturation and differentiation of dendritic cells	Notch Activation	92
	Placenta-derived MSCs	Increased placenta-derived survival and chondrogenic differentiation	Notch Inhibition	93
Primary Cells	Pulmonary artery SMCs	Inhibit proliferation, improved pulmonary hypertension	Notch Inhibition	94
	Cochlear cells	Promotes cochlear sphere formation and sensory potential	Notch Activation	95
	Coronary artery SMCs	Inhibition of neointima formation and enhanced re-endothelialization, suppressed proliferation and migration	Notch Inhibition	96
	Human foreskin keratinocytes	Maturation and differentiation of human keratinocytes	Notch Activation	97
	NIH3T3 fibroblast	Suppressed tumor onset and growth, vascularization	Notch Inhibition	98
Cell Lines	3T3-L1 adipocyte progenitor cell line	Mature adipocyte differentiation and proliferation	Notch Inhibition	99

MSCs- mesenchymal stem cells ; SMCs- smooth muscle cells.

2.6.2. Immobilized Notch ligand delivery

Due to the suspected need for anchorage to a cell or biomaterial surface, various immobilization strategies have been investigated; these include covalent immobilization, infinity immobilization, and immobilization through a flexible spacer arm. Covalent immobilization assures ligand presence, but the active site of the ligand is not always accessible for binding due to this random attachment to the surface. Therefore, Notch orientation-regulated immobilized scaffolds have been engineered using indirect affinity immobilization strategies and are the most commonly used in Notch signaling biomaterials. Antibody binding proteins such as Protein A/G, Streptavidin/Biotin binding, as well as anti-Fc antibodies, have been harnessed to control ligand accessibility and orient the ligands with the active site available for binding. Compared to covalent immobilization strategies, affinity binding strategies allow indirect immobilization, but the active site is oriented controllably for maximized receptor binding capability.

Lastly, immobilization through a flexible molecular arm has been suggested to allow for better accessibility and activity of the immobilized ligand. Optimal ligand surface coverage can be maximized with spacers due to the ability of the polymer-bound proteins to form a layer and disperse the ligands in space to optimize binding and minimize lateral repulsions[100]. To mimic the dynamic regulation of signaling ligands, polymer chemistry can be harnessed to create chemical spacers to improve biomolecular recognition, ligand accessibility and increase the dynamic behavior of immobilization[101].

A summary of Jagged1 immobilized biomaterials for vascular tissue engineering applications is shown in **Table 2-3.** Considering its role in multiple tissues, Jagged1 has been immobilized for bone tissue engineering as well as many other tissue engineering applications **(**summarized in **Appendix A2: Jagged1 biomaterial immobilization)**.

Table 2-3 Prior studies about immobilized Jagged1 for vascular tissue engineering

Authors	Year	Journal	Cell Type	Application	HES1	HEY1	Ligands	Receptors	Acta2	SM22α	Calponin	Myh11	Ref.
Benedito et al	2009	Cell	human umbilical vein ECs	tip-stalk cell selection	↑	↑	↑ Dll4						77
Xia et al.	2011	Biomaterials	human coronary artery SMCs	phenotype control				NS	NS		NS		53
Boucher et al.	2011	Journal of Biological Chemistry	aortic SMCs	phenotype control					↑	↑	↑		102
Bhattacharyya et al,	2014	Tissue Engineering Part A	human coronary artery SMCs	phenotype control				↑	NS		NS		52
Boopathy et al, 2014	2014	Biomaterials	cardiac progenitor cells	vascular differentiation		↑			↑			↑	103
Davis-Knowlton et al	2019	Laboratory Investigation	carotid artery SMCs	phenotype control				↑ Notch3	↑			NS	72
Davis-Knowlton et al	2019	Laboratory Investigation	diseased carotid artery & femoral artery SMCs	diseased phenotype recovery				↑ Notch3	NS			NS	72
Putti et al	2019	ACS Omega	human coronary artery SMCs	phenotype control	↑	↑	↑Jagged1	↑ Notch3	↑				104
Putti et al	2019	Applied Polymer Materials	vascular SMCs	phenotype control	↑	↑	↑Jagged1	↑ Notch3					105

2.7.Modulation of the Notch signaling pathway

For Notch signaling modulation, the understanding of ligand-receptor specificity is necessary to utilize developmental biology as inspiration for engineered tissues[47,106–108]. For example, the application of synthetic biology can be applied to distinguish between ligand-receptor affinity vs. avidity between various Notch ligands and receptors[107]. Nevertheless, insights into Notch modulation by analyzing the distribution and expression profiles of Notch ligands/receptors can be crucial because they vary significantly among cell types and behave uniquely to their surrounding microenvironments[34]. Within the context of this thesis the Jagged1-Notch3 pairing has been targeted for vascular applications and SMC control.

Additionally, pathway modulation occurs through the cis- and trans- ligand-receptor interaction distinction[107,109]. The balance between cis-inhibition, trans-inhibition, and trans-activation can help determine Notch-based decisions. Still, this regulatory mechanism is often overlooked as it is challenging to uncouple cis- and trans-binding properties in vivo[110]. While the response to trans-activation is gradual in response to external ligand signals on neighboring cells, cis-inhibition is a sharp immediate response, silencing when a level of intracellular ligand exceeds a threshold concentration[111]. In addition to the signaling mechanism, signaling range, cell shape, and packing geometry are also suggested to be a factor in Notch modulation[112113]. Biologically, the factors mentioned above play a role in the development and maintenance of various organs, tissues, and systems within the body. Both two-dimensional and three-dimensional microenvironments can be used to gain insight into various instructional cues apart from ligand presentation, including biomaterial cues, spatial cues, temporal cues, mechanotransduction. These cues to control Notch signaling and influence biomaterial design have been recently reviewed and published by this author[114].

2.7.1. Notch signaling as a cofactor and signaling crosstalk

In addition to the canonical Notch pathway, there is also increasing evidence that the Notch signaling pathway can be activated and modulated without its prominent role as a transcriptional cofactor[115]. It is speculated that some of the effects of Notch signaling are due to undiscovered noncanonical interactions involving various Notch components with components of various other signaling pathways. Therefore, Notch signaling might have diversity within the pathway, affecting the activity of other signaling pathways as well as utilizing signals from outside of the Notch pathway to regulate Notch activity and expression levels. Notch signaling interactions within VSMCs include Yes-associated protein/ PDZ-binding motif (YAP/TAZ**),** Platelet-derived Growth Factorβ (PDGFβ), Transforming Growth Factorβ (TGFβ), Mitogen-Activated Protein Kinase (MAPK), and Wingless-Related Integration Site (Wnt) (reviewed recently in more detail in reference[115]). A summary of crosstalk between Notch and these pathways in the vasculature is summarized (not exhaustive) in **Table 2-4.**

Despite the requirement for membrane tethering and endocytosis, the soluble ligand can activate Notch signaling in non-SEL proteins reported to be noncanonical Notch ligands[89]. Two non-DSL proteins have been identified as putative Notch ligands, including connective tissue growth factor cysteine-rich 61/nephroblastoma overexpressed gene, and microfibril-associated glycoprotein family-1,2 [89].

Table 2-4 Notch signaling crosstalk in the vasculature

Pathway	Cell source	Outcome	Ref.
YAP/TAZ	Mouse aortic smooth muscle cells	Deletion of YAP and TAZ abrogates Notch signaling in SMCs and impairs development of the aortic arch arteries	[116]
	Mouse aortic smooth muscle cells	YAP and NICD can physically interact and regulate the expression of Jagged1	[116]
PDGF-B	Human coronary artery smooth muscle cells	Notch promotes the transcription of PDGFRβ	[117]
	Human aortic smooth muscle cells	PDGF-B decreases expression levels of Notch2, but not Notch3 in SMCs	[118]
	Rat aortic smooth muscle cells	PDGF downregulates Jagged1, Notch3 and HESR-1 expression via an ERK-dependent pathway	[119]
TGFβ	Human aortic smooth muscle cells	Induces a molecular and functional contractile phenotype by co-regulation of Smad activity at SMC promoters	[36]
	Human aortic smooth muscle cells	Jagged1 promotes transcription of miR145 which inhibits TGFβ pathways to cooperatively promote actin and calponin	[102]
BMP	Human umbilical vein endothelial cells	BMP9/ALK-1 singling promotes expression of the Notch target genes Hey1 and Hey2 which inhibits VEGF-induced angiogenesis and vascular morphogenesis	[120]
	Mouse embryonic endothelial cells	BMP acts to enhance Notch in endothelial cells to inhibit migration and limit angiogenesis; increases the expression of Herp2 through formation of a transcriptional complex comprised of NICD and Smad	[121]
Wnt	Differentiated embryonic stem cells	β-catenin directly associates with and co-activates NICD forming a transcriptional complex in arterial cells regulating arterial specification	[122]
	Endothelial cells from E9.5 embryos	β-catenin promotes Notch activity inhibits migration in endothelial cells by binding to the Dll4 promoter and up-regulating the transcription of Dll4 increasing Notch signaling	[123]

PDGF-B: platelet-derived growth factor B, YAP/TAZ: Yes-associated protein/ PDZ-binding motif, MAPK: mitogen-activated protein kinase pathway, Wnt: wingless-related integration site

An important ligand for this thesis is TGFβ1. TGFβ signaling has been implicated vastly in the control and differentiation of smooth muscle cells[115]. Both *in vitro* and *in vivo* studies have shown that TGFβ signaling components are upregulated at the sites of vascular injury, detected within 6 hours of arterial balloon injury and sustained up to 14 days[124]. Additionally, TGFβ has been implicated in ECM accumulation and VSMC proliferation and migration, which are expected to counter its profibrotic effects[125]. These varying effects on VSMC are hypothesized to be related to differential but complementary signaling systems through Smad. The Smad proteins are classified into three groups; receptor-activated Smads (Smad2 and Smad3), common Smad (Smad4), and inhibitory Smads (Smad6 and Smad7). At the molecular level, these differences have been attributed to varying levels of receptor expression, membrane localization of receptors, availability of intracellular signaling mediators, and presence of transcriptional co-regulators within the nucleus[125]. Notch and TGFβ signaling have been identified as co-regulators indicating cross-talk between these pathways[38,126], and has been demonstrated in a liver fibrosis model[127]. Specific direct protein interactions have been linked in the two signaling pathways; TGFβ regulated transcription of the HES1 promoter occurs in a Notch-dependent manner, and the NICD acts cooperatively with Smad2/3[115], an intracellular transducer of TGFβ signaling as shown in **Figure 2-7.**

Activation of Smad2/3 is suggested to induce the activation of synthetic promoters containing multimerized CSL or Smad3 binding sites. The NICD and Smad3 were shown to interact directly in a ligand-dependent manner and Smad3 could be recruited to CSL-binding sites on DNA in the presence of CSL and NICD[37]. Overall, this could be useful in combination therapy approaches with promising potential to modulate phenotype control, differentiation, and other signaling in the vasculature.

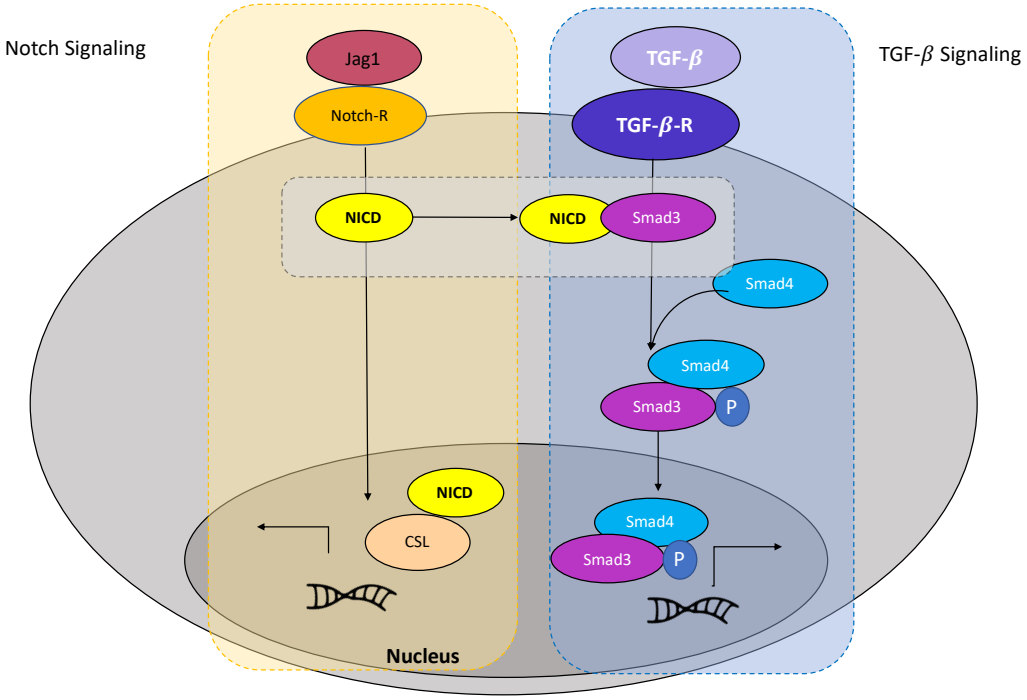

Figure 2-7 TGFβ1 Notch signaling crosstalk in VSMC control
The activation of the TGFβ receptor leads to the activation of Smad2/3, which can then intracellularly form a complex with Smad4[124]. This complex translocates to the nucleus, where it can regulate the transcription of target genes by binding to Smad-binding elements. Elements of Notch signaling can communicate and interact with Smad elements. Upon binding of the Notch ligand and receptor, the S3 cleavage generates the Notch intracellular domain (NICD) which can translocate to the nucleus or act cooperatively with Smad2/3 influencing cell behavior.

2.7.2. Notch and incorporating mechanotransduction forces

Lastly, the modulation of Notch signaling can be driven through mechanical forces. Cells respond not only to biochemical signals but also to a variety of mechanical forces. Mechanotransduction is the process of how cells convert a physical force into a biochemical signal. Cells in the body, and specifically artery walls, undergo a variety of mechanical forces within their microenvironment, whether it is from contacting cells, the surrounding tissue, or the various bodily fluids passing through the body. The response of cells to mechanical stimuli and the transmission of these forces into chemical signals (mechanotransduction) is also important to enhance Notch signaling efficacy. Cells in the vasculature are subject to various external forces, including strain

magnitude from blood pressure (upon increased or decreased wall thickening caused by vascular morphogenesis and phenotype switching)[70], and shear stress caused by blood flow (due to cycles of contraction and relaxation of heart tissue)[81,128]. Incorporating mechanical signals into biomaterial *in-vitro* systems, using bioreactors, or other platforms can allow us to understand the Notch dynamics in a system and create a better tissue-engineered design.

To further control Notch signaling, there is also evidence that Notch signaling is driven by cell-cell interactions which are mechanosensitive at the molecular level. Crystal structures have revealed the overall Notch receptor-ligand conformation indicating that the S2 binding site is deeply embedded within the Notch heterodimer LNR domain and thus is protected from metalloprotease cleavage and creates an autoinhibited conformation[129,130]. The Notch "pulling model" indicates that the Notch regulatory region of the receptor acts as a force sensor that is unfolded by a threshold level of mechanical tension generated across the ligand/ receptor bridge (**Fig. 2-8**). This tensional force is debated to be caused by the endocytosis force of the ligand-receptor complex, whereby tethering alone without ligand endocytosis was proven to be insufficient for Notch activation[131]. Notch in the absence of endocytosis, ligands were shown to accumulate on the cell surface but fail to activate Notch signaling on neighboring cells [41].

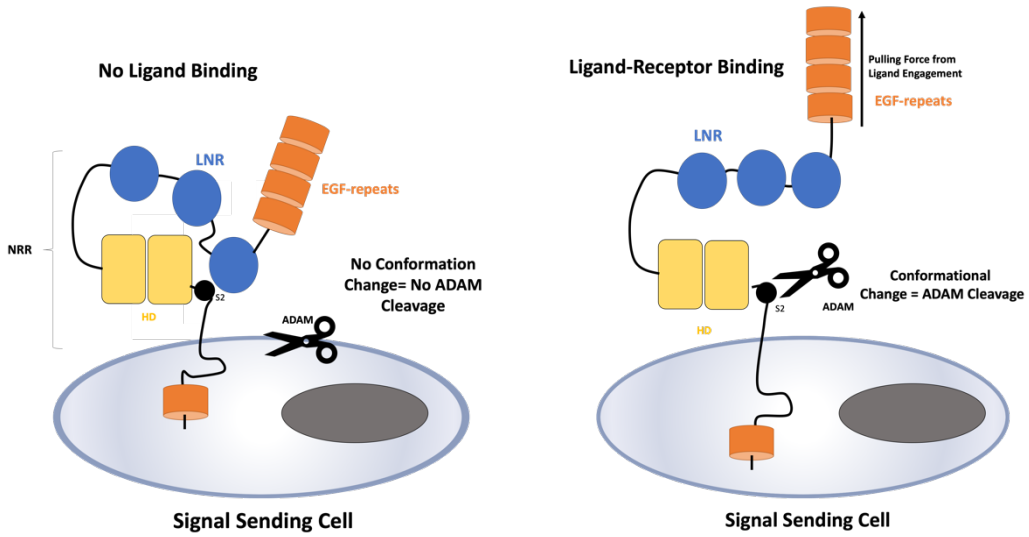

Figure 2-8 The pulling model for Notch activation
Upon ligand-receptor binding, the Notch receptor must undergo protein unfolding caused by applying a tension pulling force. A tension force at the ligand-receptor complex pulls the LNR repeats away from the S2 domain. This allows for the active site to be uncovered and S2 cleavage of the extracellular domain by ADAM to occur. Published as Kathleen Zohorsky and Kibret Mequanint. Tissue Eng. Part B. NRR-Notch regulatory region, LNR- Lin12/ Notch related domain

The role of mechanotransduction within the Notch signaling pathway is still controversial and a highly debated topic of research currently. Considering these concepts, the interplay between mechanical force transduction and its interaction with ligand immobilized to the surface of biomaterials poses another important challenge in the integration of these complex cellular processes into biomaterial-based systems. Additionally, the delivery of chemical signals via a biomaterial surface presents the absence of the mechanical tension force found biologically through the pulling force. When replacing the signal sending cell with a biomaterial surface, it is questioned whether there is sufficient molecular force (without endocytosis) for signal activation. Mechanotransduction within Notch signaling thus far has been very context-dependent; therefore, various ligand-receptor combination could prove to be more mechanosensitive than others. Up

until recently, ligand-receptor mechanosensitivity has been studied with preference to delta-like ligands and not Jagged ligands.

2.8. Molecular force recognition and force application

Various tools have been utilized to study the effects of forces on cells, including atomic force microscopy [132], optical tweezers [133], flow systems, tension gauge tethers (TGTs) [134], as well as fluorescence resonance energy transfer[135,136] tension sensors. However, biochemical analysis is difficult with many of these techniques. The use of magnetic tweezers [137–144] and magnetic beads to apply tension to cells readily facilitates both single cell assays as well as bulk chemical assays, which makes this tool readily used to study mechanotransduction. These force application and force sensing techniques are visually presented in **Figure 2-9 A, B,** respectively.

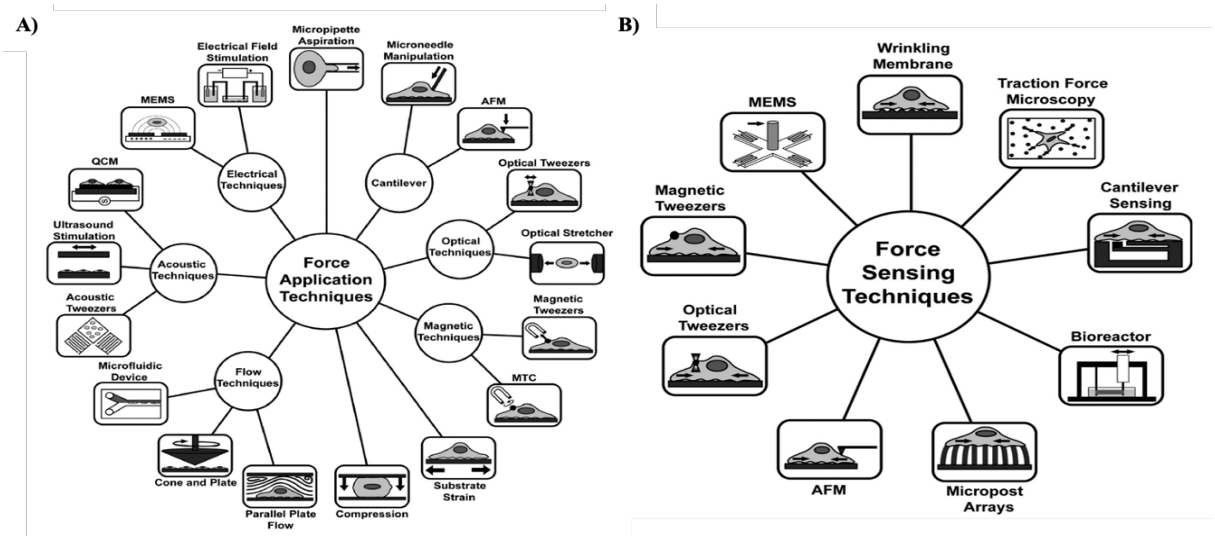

Figure 2-9 Force application and force sensing techniques
A) Force application techniques can be used to measure cell response to an applied deformation or force and can be propped by various stimulus including cantilever, optical, magnetic, fluid flow, acoustic, or electrical stimulation**. B)** Force sensing techniques are used to measure the forces produced by the cells during development, contraction, migration and other cellular processes. (Reproduced with permission from ref [145]. © Copyright 2013 by ASME)

An important tool that has been implemented to study Notch signaling is TGTs which are force sensors. ProteinG based TGTs have been frequently used to study the force magnitude

required for signal activation. Recombinant ligands with IgG-Fc fusion are assembled using DNA tethers with different tension tolerances immobilized through a glass surface passivated with PEG. The Notch ligand Dll1 was tethered to the surface and using a reporter cell line, it was determined that under 12 piconewtons (pN) of force was required for signal activation[135]. A similar result was found using another TGT assay [134]. Force-induced Notch activation has also been studied at the ligand-receptor bridge using Notch ligand immobilized to magnetic nanoparticles to form a magnetic tweezer assay. Magnets have been utilized to apply a range of pN-scale forces to the Notch receptor on the cell surface, and Dll4-loaded magnetic beads induced Notch signal activation with the addition of this mechanical tension force[131]. This further proved that force must be applied to bead-tethered ligands to further induce the canonical proteolytic steps responsible for Notch activation. Magnetic tweezers demonstrate a simple and effective strategy to introduce molecular forces in the pN and should be adapted to demonstrate mechanosensitivity in other ligand-receptor pairings as a useful way to enhance Notch signaling. These and other studies suggest Notch signaling activation might require piconewton force application that must be incorporated into biomaterial design, and thus is an interesting avenue to explore.

2.9. Thesis motivation

Proper communication between ECs and SMCs in the arterial wall is important to maintain vascular homeostasis and contractile cell behavior. Stenting is a common treatment to reopen an atherosclerotic artery. However, upon stent deployment, it causes endothelial injury resulting in reduced function and communication between these cells leading to complications including restenosis, reduced blood flow and potential heart attack. Many attempts have been made to fix the complications with stents including drug-eluting stents and biodegradable stents. These have shown little clinical success in improving restenosis. Functionalizing stent surfaces with a

bioactive protein such as Jagged1 may enable the stent to act as an endothelial cell surrogate material to direct EC regeneration, enhance contractile SMC function, and phenotype regulation of VSMCs. The long-term therapeutic objective of this project is shown in **Figure 2-10.** This figure is a schematic of a Jagged1-functionalized stent material used to regain proper arterial function and cellular communication. Short-term the effectiveness of immobilized Jagged1 delivery platforms need to be optimized, and insights into modulation of the vascular smooth muscle cell response are needed for proof of concept.

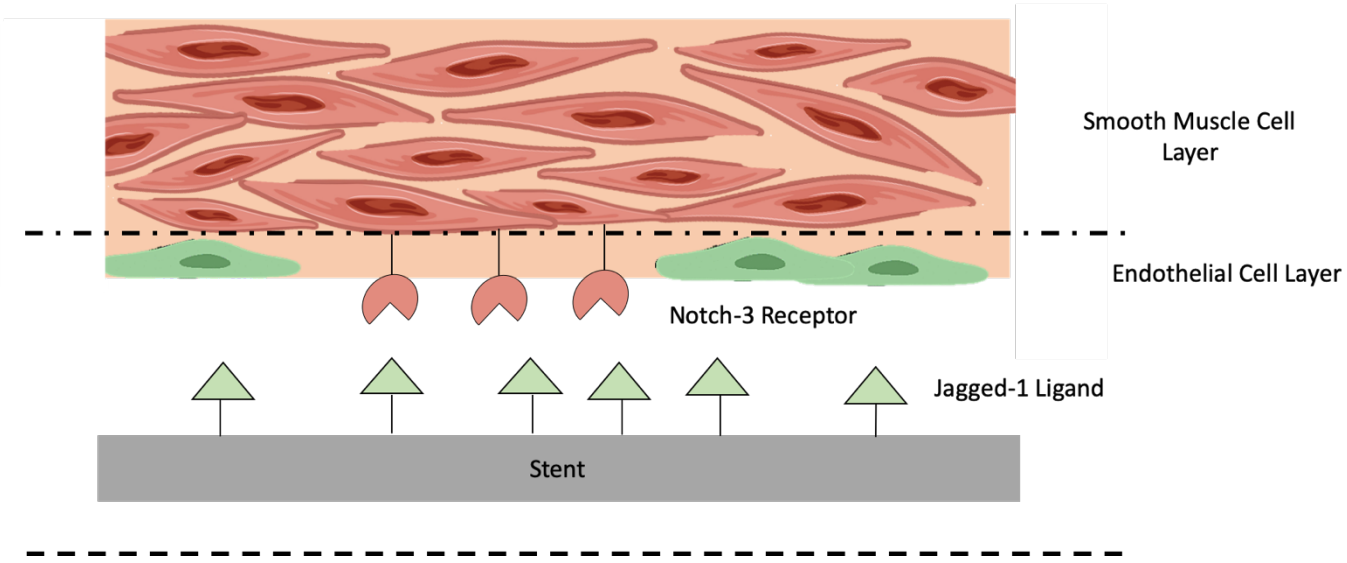

Figure 2-10 Notch signaling bioactive stent material

2.10. Thesis objectives

This thesis explored the effectiveness of Jagged1 treatment on SMC response in culture to determine the value of the proposed long-term objective. To test the signaling efficacy of Jagged1 the following specific objectives for this thesis were developed:

(i) Evaluation of the effect of Jagged1 presentation strategies on coronary artery smooth muscle cell phenotype control and differentiation

(ii) Investigation of iPSC-MSC and 10T1/2 cells for Notch signaling

(iii) Investigation of Jagged1 mechanotransduction to potentially enhance signaling efficacy

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Chapter 3. Materials and Methods

This chapter details the methodologies used to direct smooth muscle cell differentiation and phenotype control utilizing Jagged1 directed Notch signaling.

3.1.Materials

Proteins. Protein G Dynabeads™ were purchased from Invitrogen (Burlington, ON, Canada) to immobilize recombinant human Jagged1/Fc chimera Protein (1277-JG) which was purchased from R&D Systems and reconstituted in Phosphate-buffered saline (PBS). For Notch signaling studies, the Notch inhibitor DAPT was purchased from Sigma. Transforming growth factor-beta 1 (TGFβ1) was purchased from Abcam (Cambridge, MA), and Fibronectin (FN) was supplied by Santa Cruz Biotechnology (Sant Cruz, CA) to promote cell adhesion. Hanks' Balanced Salt Solution (HBSS) used for solubilizing FN and washing cells which was purchased from Gibco (Maryland, USA).

Cells and Cell Culture Media. Cell culture studies were conducted using primary human coronary artery smooth muscle cells (HCASMCs) cultured in smooth muscle growth media (SmGM; SmGM®-2 BulletKit) obtained from Lonza (Walkersville, MD). Media was supplemented with 0.50 mL insulin, 1.00 mL hFGF-B, 0.50 mL GA-1000, 25mL FBS, and 0.5mL hEGF, as provided in the SmGM-2 SingleQuots Kit. Other cell types used were mouse embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) purchased from ATCC and maintained in Dulbecco's modified Eagles medium (DMEM) (ThermoFisher) containing 5% fetal bovine serum (FBS) (Thermo Fisher) and 1% penicillin/streptomycin (ThermoFisher) by volume. Lastly, iPSC-derived MSCs were a gift by Dr. Dale Laird (Western University, Canada). iPSC-MSCs were maintained in Mesenchymal Stem Cell Expansion Media (MSCEM, Cellular

Engineering Technologies Inc., IA, USA), supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin/streptomycin (all from Life Technologies, Canada).

Additional Materials for Mechanotransduction Studies: Sylgard 184 silicone elastomer pre-polymer and the curing agent (PDMS, Part A and B) were used to change the culture well height of the 96-well plate and was purchased from Ellsworth Adhesive Chemical Co. A plate containing 96 cylindrical magnets used to provide a magnetic force to the Protein G beads was purchased from Alpaqua Engineering.

Western Blot: The antibodies for use in Western blot (anti-Acta2, anti-Cnn1, anti-Myh11, anti-Jagged1, anti-Notch3) were purchased from Santa Cruz Biotechnology with the exception of GAPDH which was purchased from Millipore (Temecula, CA). Protein concentrations were measured using 660 nm Protein Assay supplied by Thermo Scientific (Ottawa, Canada). SuperSignal® West Pico Chemiluminescent Substrate was supplied by Thermo Scientific (Rockford, IL).

RT q-PCR: RNA analysis of HCASMCs was achieved using TRIzol® Reagent and SuperScript™ from Invitrogen and a Chromo4 Real-time Thermal Cycler, iQTM SYBR® Green Supermix and Gene Expression Macro analysis software from Bio-Rad (Mississauga, ON, Canada).

Immunofluorescence: For cell fixation, paraformaldehyde was purchased from EMD Chemicals. (Gibbstown, NJ). All Alexa-594 conjugated goat anti-mouse and Alexa-594 conjugate goat anti-rabbit antibodies were purchased from ThermoFisher Scientific. Additionally, the Alexa™ Fluorr 594-conjugated phalloidin to stain F-actin was also purchased from ThermoFisher Scientific. A Zeiss LSM 510 confocal microscope is from Zeiss, Canada .

3.2. Cell culture

3.2.1. Primary human coronary artery smooth muscle cells (HCASMCs)

Primary HCASMCs were cultured in smooth muscle growth media (SmGM). Cell cultures were maintained in a humidified incubator at 5% CO_2 and 37 ^oC and were used between passages 4-11.

3.2.2. Human-induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSC)

IPSC-MSCs were grown on gelatin (Sigma-Aldrich) coated dishes in Mesenchymal Stem Cell Expansion Media (MSCEM). Culture dishes and plates were coated pre-coated with 0.1% gelatin solution (Signa-Aldrich) for 1 hr. Media were changed every other day for until confluency. Cell cultures were maintained in a humidified incubator at 5% CO_2 and 37 ^oC and were used between passages 8-12.

3.2.3. Mouse embryonic multipotent mesenchymal progenitor (10T1/2) cell line

Undifferentiated embryonic multipotent mesenchymal progenitor cell line, 10T1/2 cells were maintained in modified Eagles' medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin by volume. 10T1/2 cells treated with 2ng/mL TGFβ1 for a period of three days were used to pre-differentiate these cells towards a vascular lineage. Media were changed every three days, and cells were passaged when they reached 90% confluency as per manufacturers recommendations. Both undifferentiated and predifferentiated 10T1/2 cells were used for future experiments. (Differentiation protocol from ref [2]).

3.3.Jagged1/Fc protein immobilization to Protein G magnetic Dynabeads™

The maximum binding capacity of Protein G beads provided by the manufacturer states that 100 µL of Protein G Dynabeads™ will isolate approximately 25-30µg of human IgG per a sample containing 20-200µg/mL. Protein G Dynabeads at a volume of 200 beads/cell were washed 3 times with PBS (pH 7.4, 0.02% Tween). The addition of 2.5 µg/mL (culture media) of human Jagged1/Fc chimera protein (original concentration 200 µg/mL) was added to the Protein G bead suspension and incubated for 10 minutes under rotation at room temperature. To remove the unbound Jagged1 the immobilized beads were washed 3X with PBS. Beads were resuspended in PBS and added to cell cultures at a concentration of 200 beads/cell. The immobilization scheme is shown in **Figure 3-1.**

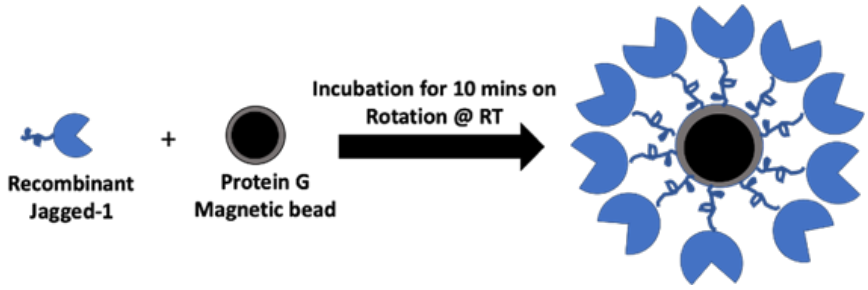

Figure 3-1 Jagged1 immobilization scheme to Protein G beads

3.4.Jagged1 delivery to control smooth muscle cell differentiation and phenotype control

Cells were seeded in culture dishes and incubated for 48 hours to allow for cell attachment. Cells were treated with ProteinG beads (200 beads/cell), soluble Jagged1 (2.5µg/mL), or Jagged1 immobilized beads (2.5 µg/mL, 200 beads/cell), to determine the effects of Jagged1 delivery on SMC response (Protocol adapted from ref [3,4]). This was done for HCASMCs and iPSC-MSCs. For

10T1/2 cells, the effect of both soluble and immobilized Jagged1 was analyzed for both undifferentiated and TGFβ1 pre-differentiated cells.

3.4.1. Serum starvation of HCASMC

Cover slides were coated with 0.1% gelatin for 1 hr at 37 ºC. HCASMCs were seeded on coverslips at a density of 2 500 cells/cm^2 and cultured until cells reached 50% confluency. HCASMCs maintained in SmGM were used as controls. HCASMCs were cultured in serum-free DMEM, and in serum-free DMEM + TGFβ1 (2ng/mL) for 72 hours. 10T1/2 cells were then fixed and stained with anti-calponin1/2/3, anti-smoothelin, and anti-Myh11, and imaged using confocal microscopy to look at protein expression levels and morphological changes.

3.4.2. Notch inhibition with DAPT

Cells were plated and cultured for 48-hours and treated overnight with 10μM DAPT (a γ-secretase Notch inhibitor). The spent media was aspirated and replaced with fresh media, and cells were treated with an additional 10μM DAPT ± Jagged1 immobilized beads (2.5μg/mL,200 beads/ cell). The DAPT inhibition assay was performed for HCASMCs, iPSC derived MSCs, and pre-differentiated 10T1/2 cells. Cells cultured alone were used as controls for these experiments. Expression of downstream gene and protein targets were done by RT-qPCR, Western Blot, or immunofluorescence microscopy.

3.4.3. Notch and TGFβ crosstalk

To examine the effects of Jagged1 and TGFβ1 ligands on SMC response, HCASMCS were plated in 24-well culture dishes at a seeding density of 50 000 cells/ well and cultured for 48 hours to allow for cell spreading and cell attachment. Cells were treated with i) Jagged1 immobilized beads (2.5μg/mL, 200 beads/cell) ii) Jagged1 immobilized beads (2.5μg/mL, 200 beads/cell) +

DAPT (10mM) iii) TGFβ1 (2ng/mL), iv) TGFβ1 (2ng/mL) + DAPT, and v) Jagged1 immobilized beads 2.5μg/mL + TGFβ1 (2ng/mL). All cell treatments were done for a 3-day timeframe. DAPT was used to determine Notch specific response, and the combination treatment was used to demonstrate potential crosstalk between the two signaling pathways. Cells cultured alone were used as controls for these experiments. The cell response was analyzed by qPCR.

3.5. Exploring Jagged1 mechanotransduction to potentially enhance signaling efficacy

HCASMC's or pre-differentiated 10T1/2 cells were seeded in a 96-well plate at a seeding density of 10 000 cells/well and cultured for 48 hours to allow attachment and cell spreading. Cells were cultured for 12 hours with the addition of immobilized Jagged1 (2.5 μg/mL, 200 beads/cell). Cells were left overnight to allow for receptor-ligand binding, then a multiplexed cylindrical magnet plate (**Fig. 3-2 A)** was positioned over the 96-well plate of cells (1 magnet per well). This applied a tension force to the magnetic beads bound to the Notch3 receptors on the signal receiving smooth muscle cell (**Fig. 3-2 B**). Cells were cultured for 3 days before cells were analyzed with RT-qPCR. Results were compared to cells in the presence of Jagged1 immobilized magnetic beads without the application of a magnetic tension force, and cells without the Jagged1 beads ± the 96-well magnet (Protocol adapted from [6])

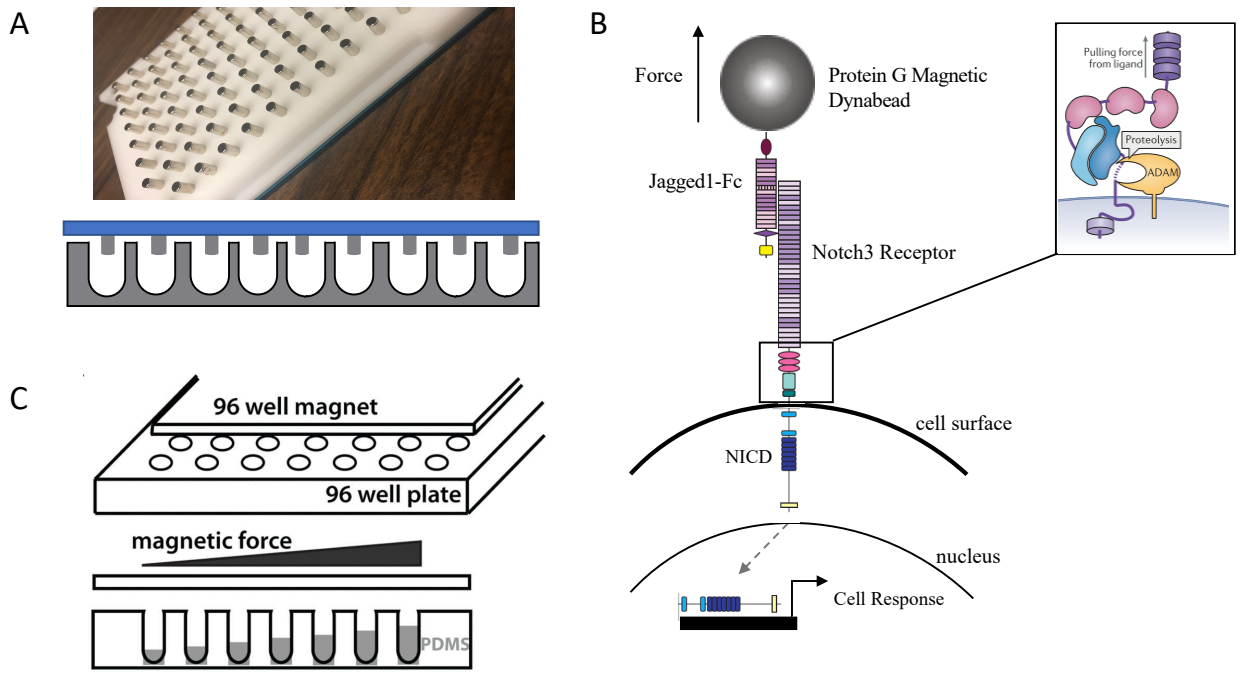

Figure 3-2. 96-well magnetic tweezer setup.
A) 96 well cylindrical magnet plate set-up. **B)** Force application to magnetic Dynabeads™ tethered to Notch receptors on the smooth muscle cell surface, and subsequent ADAM S2 cleavage (Adapted with permission from ref [5] Copyright © 2016, Springer Nature, and ref [6], Copyright © 2015 Elsevier Inc.) **C)** Terraced magnet configuration with PDMS Polymer to control force magnitude. (Reproduced with permission from ref [6], Copyright © 2015 Elsevier Inc.).

In order to explore various piconewton force magnitudes, the distance between the cells and magnet was varied using PDMS polymer to create terraces of different heights (**Fig. 3-2 C**) (Protocol was adopted from ref [6]). PDMS of various volumes, 0 µL, 60µL, 90µL, 120µL were dispensed into a 96 well plate (increasing the height increases the tension force applied on the ligand-receptor complex). PDMS surface was sterilized with 70% ethanol at RT for 1 hr, followed by FN adsorption for 1 hour at 5 µg/cm^2. Cells were plated at a density of 10 000 cells/well and cultured for 48 hours to allow for cell attachment and growth. Next cells were treated with Jagged1-immobilized Dynabeads™ (2.5 µg/mL, 200 beads/cell) for 12 hours. After 12 hours, the magnet plate was added on top of the culture dish and cultured for an additional 48 hours.

HCASMC cultured alone, and HCASMCs cultured with Jagged1 immobilized Dynabeads™ without a magnet lid served as controls.

3.6. RNA isolation and quantitative real-time quantitative polymerase chain (RT-qPCR) analysis

Total RNA was isolated from 2D cell cultures using TRIzol. Spent media was aspirated and 1 mL TRIzol per 10 cm^2 was used per treatment group to collect cells using TRIzol reagent (Invitrogen, USA). Cells were pipetted several times to form a homogenous lysate then were left at room temperature for 10 mins to allow for complete dissociation of nucleoprotein complexes. 500μL of chloroform was added, vortexed for 15 sec, then incubated at RT for 15 min. The samples were centrifuged at 12,000 x *g* for 15 min at 4°C. The organic phase was discarded, and the aqueous phase was transferred to a fresh 2mL Eppendorf tube. Isopropanol was added at a ratio of 1:2 (isopropanol: TRIzol) and incubated at RT for 10 mins followed by centrifugation at 12,000 x *g* for another 10 mins at 4°C. Isopropanol was then aspirated from the Eppendorf tube, and the RNA pellet was resuspended in 1 mL of 75% EtOH. Lastly, the suspended pellet was centrifuged 7500 × *g* for 5 min at 4 °C. EtOH was aspirated and the RNA pellet was air-dried for 10 mins in the fume hood. The RNA pellet was dissolved in 25 μL of DEPC water and quantified with the Nanodrop reader (Thermo Scientific). 1 μg of total RNA was used to synthesize cDNA using M-MLV reverse transcriptase kit (Promega) using the supplier's protocol. Reverse transcription was performed as per the manufacturer's instructions.

To prepare RT-qPCR reactions, 1 μL of cDNA was used per 10 μL reaction using the SsoAdvanced universal SYBR green Supermix (Bio-Rad) according to the manufacturer's protocol. Quantitative real-time PCR was conducted in 10 uL reaction volumes, using a Chromo4 Real-time Thermal Cycler, and gene expression of human Jagged1, Notch3, HES1, SM-α-actin

(*Acta2*), calponin (*Cnn1*), myosin heavy chain (*Myh11*) and glyceraldehyde- 3-phosphate dehydrogenase (*Gapdh*, reference gene). Gene expression was then determined with iQTM SYBR® Green Supermix according to the recommended manufacturer protocol. For HCASMCs and iPSC-MSC cells, human-specific (**Table 3-1**) forward and reverse primer sequences were used for amplification. Furthermore, for 10T1/2 cells, mouse-specific forward and reverse primer sequences (**Table 3-2**) were used for amplification. The RT-qPCR reactions were carried out in a CFX96 Real-Time thermal cycler (Bio-Rad) and *Gapdh* was used as a reference gene.

Table 3-1.Primers for human-specific mRNA amplification.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
HES1	GCACAGAAAGTCATCAAAGCC	CGCGAGCTATCTTTCTTCAGA
Acta2	CAAGTGATCACCATCGGAAAT G	GACTCCATCCCGATGAAGGA
Cnnl	TGAAGCCCCACGACATTTTT	GGGTGGACTGCACCTGTGTA
Myh11	*GACCAGGATCTCATCCTCCA*	*AGCAGCTACAGGCTGAAAGG*
Gapdh	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT

Acta2- smooth muscle-α-actin; *Cnn1*- calponin; *Myh11*- smooth muscle myosin heavy chain 11

Table 3-2 Primers for mouse-specific mRNA amplification.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
Jagged1	*TGCGTGGTCAATGGAGACTCCT*	*TCGCACCGATACCAGTTGTCTC*
Notch3	*GGTAGTCACTGTGAACACGAGG*	*CAACTGTCACCAGCATAGCCAG*
HES1	GGAAATGACTGTGAAGCACCTCC	GAAGCGGGTCACCTCGTTCATG
Acta2	*GGGCTATATAACCCTTCAGCG*	*GCTGTCTTCCTCTTCACACAT*
Cnnl	*ACTGGGTACAGATCAGCCTCT*	*TAGGCAGAGTTGTAGTAGTTG*
Myh11	*CTGGTTACATTGTAGGTGCCA*	*GCGAGCAGGTAGTAGAAGATG*
Gapdh	*AAGGGCTCATGACCACAGTC*	*GTGAGCTTCCCGTTCAGCTC*

Acta2- smooth muscle-α-actin; *Cnn1*- calponin; *Myh11*- smooth muscle myosin heavy chain 11

3.7. Immunofluorescence microscopy

For immunofluorescence imaging, cells were seeded on either 6-well plates containing a circular coverslip (area 1.9 coverslip cm^2), or a 35mm culture dish containing 2 square coverslips (area of 1.9 cm^2). Coverslips were sterilized using ethanol. Fibronectin (FN) was diluted in HBSS to a desired concentration of 5μg FN/cm^2 and absorbed onto coverslips for 1 hour at room temperature to improve cell attachment. After 24 h, HCASMCs, or 10T1/2 cells were treated with various treatments as described in the protocols above. After treatment for 72 hours, cells were washed with PBS and fixed using a 4% solution of paraformaldehyde for 30 mins at room temperature. Next, cells were permeabilized in 0.5% (v/v) Triton X-100 in PBS for 15 min and washed three times with 1x PBS. Finally, cells were blocked with 5% BAS in PVS-T for 1 hr. Blocking solution was aspirated and 100 uL of the appropriate antibodies (mouse anti-Acta2 (1:100), mouse anti-calponin1/2/3 (1:100), rabbit anti-smoothelin, (1:100), mouse anti-Myh11 (1:100)) in 5% BSA PBS-T covered overnight at 4 ^oC. Cells were washed 2X with PBS-T and 1X with PBS, and then primary antibody binding was detected by incubating cells with the corresponding secondary antibody (Alexa-488 conjugated goat anti-mouse and Alexa-594 conjugated goat anti-rabbit (1:150)) in 5% BSA PBS-T for 1 hr. at RT. 4′,6-Diamidino-2-phenylindole (DAPI; 300 nmol in PBS) was used to visualize cell nuclei, was used and F-actin was stained with Alexa™ Fluor 594-conjugated phalloidin (1:100). Images were taken with a Zeiss LSM 510 confocal microscope (Zeiss, Canada) equipped with an argon/neon as well as a UV laser. Quantification of the fluorescence intensity was performed using the ImageJ software. The target protein was quantified and normalized to the control.

3.8. Western blotting

Western blot was used to look at protein expression levels of Jagged1, Notch3, SM-α-actin, and calponin in 2D cultures. Cells were washed 3X with 1mL/well of ice-cold phosphate-buffered saline (PBS), and then harvested with 150 μL/well of ice-cold NP-40 Lysis buffer with protein inhibitor to extract whole cell lysate. Cells were kept on ice for 15 mins to allow for complete cell lysis. The cell suspension underwent three freeze-thaw cycles in the -80ºC freezer. Lysates were micro-centrifuged at 12 000 RPM for 15 mins. The pellets were discarded, and the total protein concentrations found in the supernatant were determined using a Pierce BCA protein 562nm colorimetric protein assay according to the manufacturer's instructions.

Twenty micrograms per well of protein was loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 60 min and then subsequently transferred onto a nitrocellulose membrane. Ponceau S stain was used to verify the proper protein transfer to the membrane, then was washed with DI water to de-stain the membrane. Membranes were blocked with 5% nonfat dry milk in 1XPBS for 1 h and then incubated in primary antibodies diluted in 5% nonfat dry milk in 1×PBS. The membrane was incubated for 2 h with primary antibodies, which included anti-SM α-actin (1:1000 dilution), anti-calponin (1:1000 dilution), anti-Jagged1 (1:200 dilution), anti-Notch3 (1:200 dilution), anti-GAPDH (1:2000 dilution, reference gene), all from Santa Cruz, Inc. Next the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. To image the membranes, they were incubated for 5 min in SuperSignal®West Pico Chemiluminescent substrate. Bio-Rad's ChemiDoc™ XRS+ System was used to image the membranes and blots were quantified using Image Lab™ software.

3.9. Statistical analysis

Data are presented as the relative means ± SD and normalized to the experimental control of cells plated alone. Statistical significance was calculated either using student's t-test or one-way ANOVA followed by Tukey's post hoc test to compare differences between groups. Values of $p<0.05$ were considered to be statistically significant.

3.10. References

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Chapter 4. Results and Discussion

This chapter presents and discusses the results of various experiments to evaluate the influence of Jagged1 on the control of vascular smooth muscle cells.

4.1. Jagged1 directed control of Notch activation and phenotype control in HCASMCs

In this study, the effect of Jagged1 on smooth muscle cell phenotype control driven through the Notch signaling pathway was studied. Notch signaling has been suggested to control both developmental and mature vascular tissue; specifically, the Notch ligand Jagged1 has been proven to drive phenotypic modulation in smooth muscle cells[1–3]. Jagged1 a Notch ligand, is a transmembrane protein expressed predominately in vascular ECs in the arterial wall[1,4]. The activation of the Notch3 receptor by Jagged1 maintains an autoregulatory, positive feedback loop by which Jagged1 robustly induces Notch3 expression to maintain a differentiated phenotype[5]. The Notch receptor-ligand family and domain organization of the proteins are shown in **Figure 4-1.**

As previously determined, endothelial cell-bound Jagged1 has shown promising results in coculture models to control the contractile phenotype of HCASMCs[3,6,7]. Furthermore, endothelial-specific knockout of Jagged1 has resulted in improper embryonic development and the absence of smooth muscle gene expression in the vasculature[1]. Given that Notch signaling is suggested to be driven by contact specific cell-cell communication, one goal of the study was to recapitulate the EC-SMC relationship by developing a bead-based EC-surrogate.

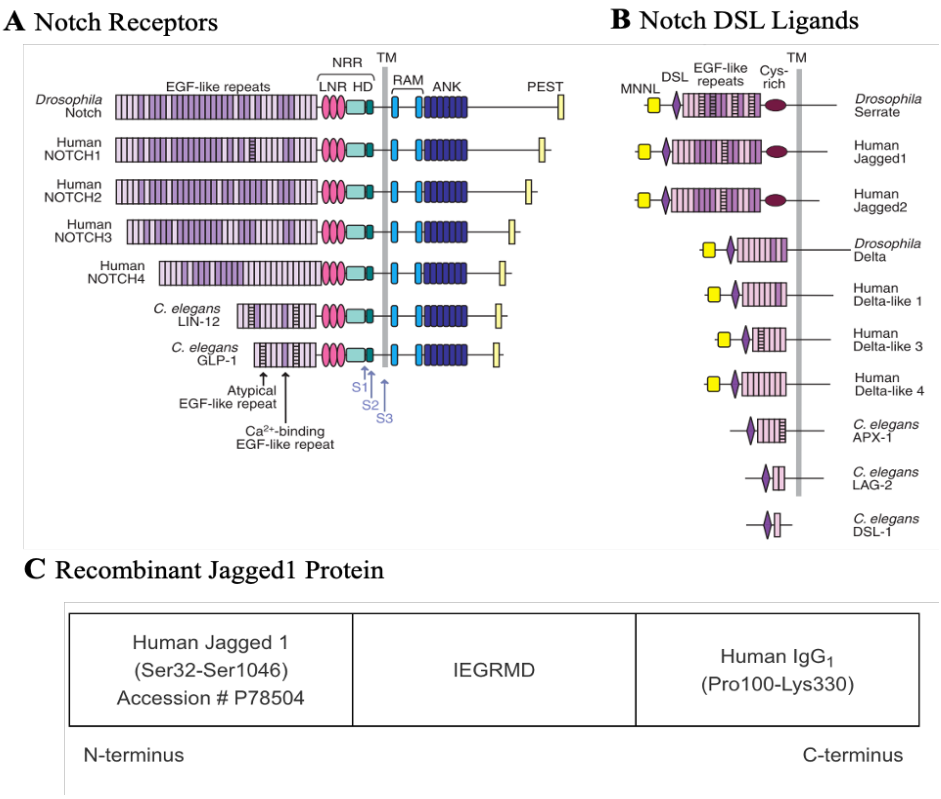


Figure 4-1 Domain organization of Notch ligands and receptors.
A) Notch receptor domain organization **B)** Notch DSL ligand domain organization **C)** Recombinant Jagged1 domain organization. TM- transmembrane, LNR-Lin-12/Notch repeat, HD-heterodimerization domain.

From a tissue engineering or therapeutic perspective, soluble Jagged1 could be delivered to cells via the culture medium if it induces HCASMCs contractile phenotype. In contrast, immobilized Jagged1 delivered via surface immobilization to biomaterials or beads may also be an approach to produce a significant influence on cell contractile phenotype driven by Jagged1-Notch3 signaling. The rationale behind surface immobilization rather than embedded delivery is that the bead surface could potentially mimic the signaling cell surface by presenting Jagged1 to adjacent HCASMCS and allow for direct cell-bead contact. Furthermore, this may allow proper cellular pulling or traction forces needed for Notch activation. **Table 4-1** provides a summary of the use of Jagged1 bead immobilization to direct cellular responses. This thesis will focus on

Jagged1 presentation strategies, including both soluble and bead immobilized delivery of Jagged1 to direct and control SMC phenotype.

Table 4-1 2D immobilization strategies of Notch ligands to microbead systems

Platform	Immobilization Method	Notch Ligand	Application	Cell Type	Ref.
Bead Immobilization	Biotin Linker	Dll4	Hematopoietic differentiation into T cells, myotube inhibition	C2C12 (mouse) myoblasts	[8]
	Protein G	Dll4	Micropatterning angiogenesis	HUVECs	[9]
		Jagged1	Smooth muscle cell differentiation and phenotype control	Human coronary artery smooth muscle cells (HCASMCs)	[6,7]
		Jagged1	Osteogenic differentiation	Human MSCs	[10]
	SNAP Tag Sequence	Dll1	Force activation of Notch receptors	U2OS cell line	[11]
	EDC/NHS + antipolyhistidine	Jagged1	Biphasic effect on cardiac differentiation, ectodermal differentiation	Human embryonic stem cells (hESCs)	[12]

4.1.1. Jagged1 presentation strategies for HCASMC differentiation and phenotype control.

HCASMCS were cultured for 36 hours in media containing 2.5 μg/ml soluble Jagged1 or 2.5 μg/ml immobilized Jagged1 (200 beads/cell). Protein G beads at a concentration of 200 beads/cell were used as a control to account for any nonspecific effects of Protein G on HCASMC response. Protein G was selected as the immobilization method because it allows controlled immobilization

of recombinant proteins through the Fc domain of the ligand, which would orient Jagged1 with the active site available for binding. Although affinity immobilization forms a non-covalent bond trapping the ligands to the surface using various proteins, the binding strength of Protein G (k_d~ 10^{-10}) is sufficient to withstand cellular forces that may break the bond, thus stable in culture conditions. Orientation-regulated immobilization enables to optimize the number of ligands available on the surface for binding to Notch receptors. The maximum capture efficiency of Protein G is approximately 0.25 μg human igG per 1μL bead volume, in its original bead concentration (as provided by the manufacturer). To immobilize Jagged1 to the bead surface, recombinant Jagged1 was incubated with ProteinG magnetic Dynabeads™ under rotation for 10 min at room temperature.

Previously studies suggested that SMC were not responsive to the IgG control which accounts for non-specific effects of the Fc-fragment found on the Jagged1 protein [6,7]. In order to assess the role of soluble and immobilized Jagged1 stimulation on HCASMC Notch3 signaling, multiple smooth-muscle cell markers were measured and analyzed **(Figure 4-2),** including *HES1* a Notch transcription factor and contractile SMC markers SM-α-actin (*Acta2*), Calponin (*Cnn1*) and Myosin heavy chain (*Myh11*).

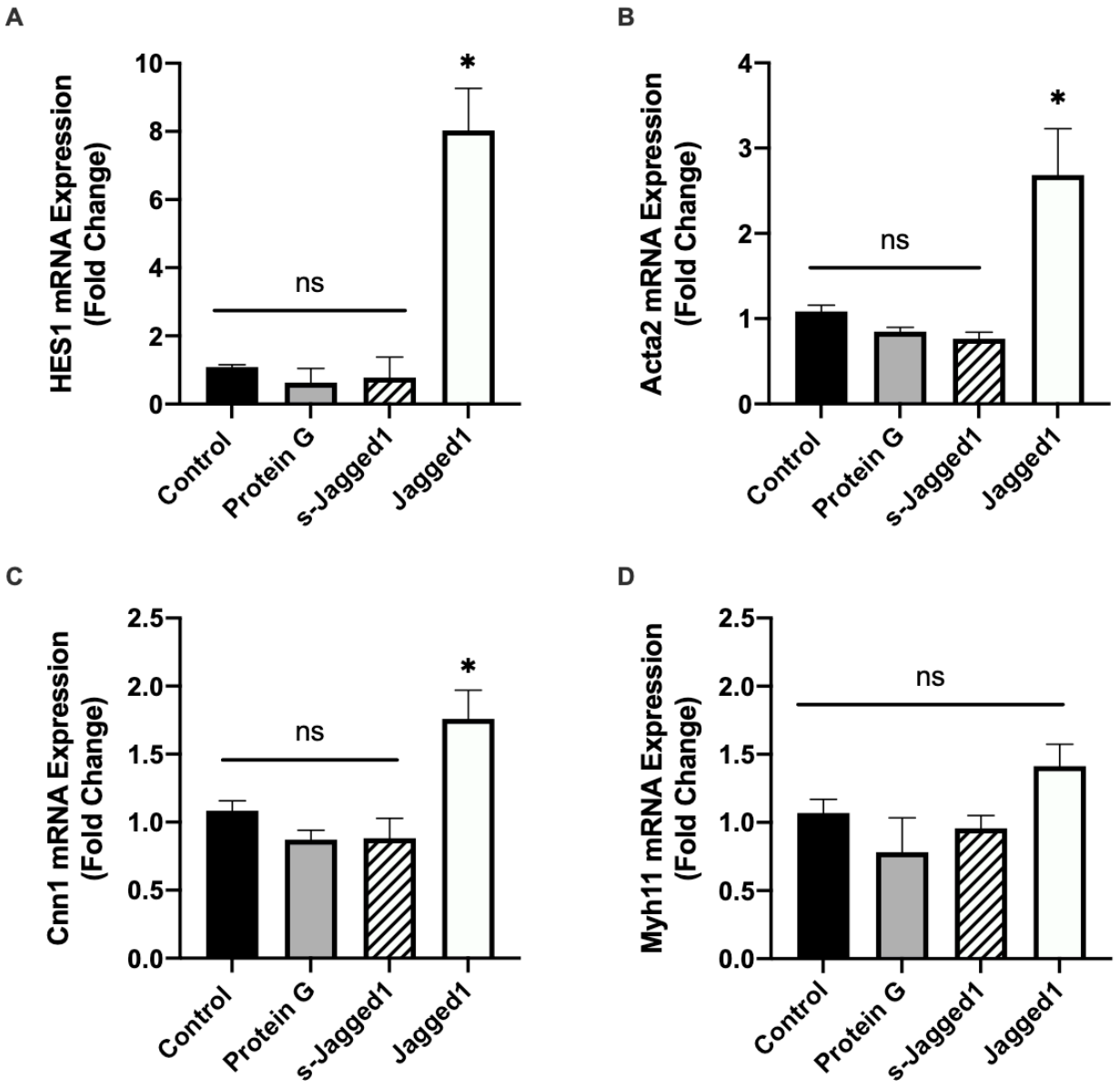


Figure 4-2 Immobilized Jagged1 upregulates Notch transcription factor and early-stage smooth muscle cell contractile markers in HCASMCs.
HCASMCs were cultured for 2 days in 24 well plate, and then incubated for 3 days with i) soluble Jagged1 (2.5µg/mL), ii) Protein G beads (200 beads/cell) or iii) immobilized Jagged1/Fc beads (2.5µg/mL, 200 beads/cell). Expression levels of **A)** *HES1, **B)** Acta2*, **C)** *Cnn1*, and **D)** *Myh11* of 3 independent studies were quantified by RT-qPCR and compared to untreated HCASMC cultures in SmGM (represented as the control). Jagged1 immobilized beads were able to upregulate the expression of both the transcriptional factor *HES1* and contractile marker genes *Acta1* and *Cnn1*, with no significant effect on *Myh11*. Data are represented as mean ± SD, normalized to the control. * indicates significance $p < 0.05$.

4.1.1.1. Soluble Jagged1 delivery on smooth muscle cell response

Although delivery of soluble Notch ligands primarily acts as an inhibitor of the Notch signaling pathway[13–19], its delivery has been therapeutically useful in certain applications to control cell fate decisions[20–24] (refer to **Table 2-2, Section 2.6.1**). With support from the literature, the data suggests that the addition of soluble Jagged1 into HCASMC cultures did not significantly affect the expression of *HES1* or any smooth-muscle contractile markers measured (**Fig. 4-2 A-D**). Since soluble Jagged1 failed to upregulate SMC contractile marker genes, it is reasonable to infer that similarly to cis-ligand interactions which are inhibitory, soluble Jagged1 also may not be able to activate Notch3 receptors. This may be attributed to the lack of cellular force available at the ligand-receptor complex to create the conformational change and expose the S2 cleavage site[26].

4.1.1.2. Immobilized Jagged1 delivery on HCASMC gene expression

Since soluble Jagged1 was insufficient to induce SMC contractile function, immobilized Jagged1 was investigated. The immobilization of Jagged1 significantly enhanced the transcription factor *HES1* as well as early and mid-stage SMC contractile markers SM-α-actin (*Acta2)* and Calponin (*Cnn1)*. Results from **Figure 4-2 A** indicate a significant upregulation in the presence of Jagged1 beads ($p<0.05$). These results support that activation of the Notch signaling pathway was driven in response to bead-bound Jagged1. HES1 expression is caused when the intracellular domain of Notch3 is released from the plasma membrane post S3 cleavage. The association with RBPJ causes conformation changes of proteins within the nucleus which also for co-activators to bind, and this new activating complex promotes HES1 expression. Consistent with previous studies where immobilized Jagged1 significantly upregulated Notch3 gene expression[7], the current study provides additional support for the upregulation of contractile gene markers. Gene

expression of early/mid-stage SMC contractile markers, *Acta2,* and *Cnn1* were also upregulated (**Fig. 4-2 B, C**).

SM-α-actin (SMA, *Acta2)* has been previously linked as a direct target of Notch/CSL domain; CSL directly binds to a conserved cis-element in the SMA promoter and this is required for Notch-mediated SMA induction[27]. From this study, *Cnn1* also seems to be linked with the Notch signaling pathway directly. A HES1 site in the promoter of the Cnn2 isoform of calponin has been associated as a tension-regulated (substrate stiffness) repressor responsive to Notch signaling[28]. Additionally, other studies have postulated that Notch induction of Cnn1 is dependent on Notch-CBF1 activity[29].

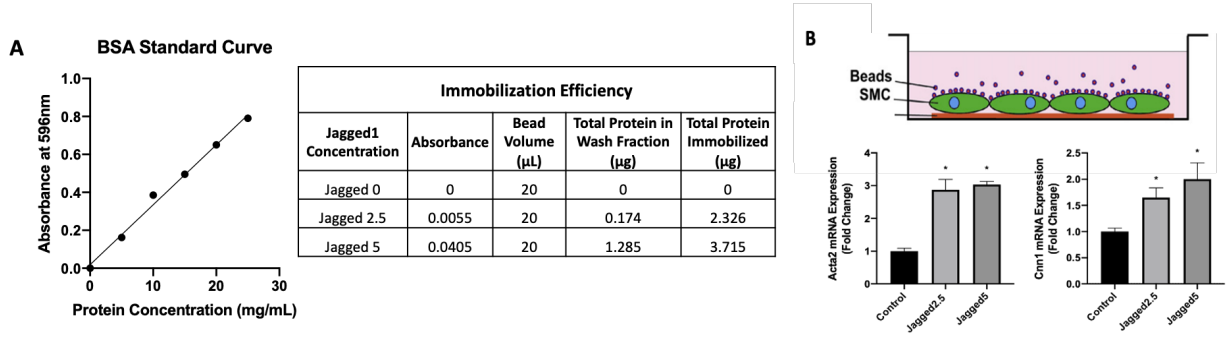
Lastly, myosin-heavy chain expression (*Myh11*), a late-stage contractile marker, was not significantly affected in these cells over a 3-day culture period (**Fig. 4-2 D)**. This marker has not been linked as a direct target of Notch signaling in the literature thus far for vascular smooth muscle cells. Longer culture times should be investigated in the future to determine if SMCs in an extended Jagged1 activated state may start to produce later stage contractile marker myosin heavy chain. Overall, this data (**Fig. 4-2)** suggests that to mimic the proper cell-cell communication needed for Notch signaling, driven by juxtacrine interactions, the immobilization of Jagged1 to a surface is needed. Therefore, it is suggested that immobilization can provide the structural cues necessary for HCASMC Notch activation and phenotype modulation.

4.1.1.3. Jagged1 immobilization concentration dependence of HCASMCs

Because Notch signaling is stoichiometrically driven rather than enzymatically driven, there is likely a concentration dependency of Notch signaling on cell response. The concentration/dose dependence and time-dependent response of cells have been shown for stem cell osteogenic differentiation[10,30], and cochlear stem/progenitor sphere formation[24]. However, dose-response seems to be context-dependent. Previously Jagged1 concentration dependence on HCASMCs was not seen[6] while dose dependency has been demonstrated for other Notch ligands such as Dll1[31]. Thus, the influence on Jagged1 concentration was investigated further. Based upon the literature, there is a wide range of Jagged1 concentrations for bead immobilization ranging from 0.5-10 μg per sample immobilized in 100 uL-150uL bead volumes per treatment group[9].

In this study, the bead volume was kept constant at 20 μL Protein G xbeads and 0-5 μg of Jagged1 was immobilized to the bead surface and subsequently cultured with HCASMCs. An ELISA assay was used to determine total immobilized protein by using the bead wash fractions and measuring the total protein that is not immobilized to the bead surface (**Figure 4-3 A)**. The protein measured in the wash fraction was then subtracted from the total protein concentration added to determine the total protein amount immobilized to 20 μL of beads. As per the manufacturer's instruction, 5 μg is the maximum concentration per this specific volume. We did not reach the maximum binding capacity (**Figure 4-3 A)** of these beads as reported by the manufacturer, but this could be due to steric hinderance, and may be improved by extending Jagged1 incubation time up to 120 mins as given by the Protein G bead manufacturer guidelines and as done in some protocols[9,32,33]. **Figure 4-3 B** shows the dose-dependent response of HCASMCs by increasing Jagged1 pre-coat concentration, using RT-qPCR gene expression of *Acta2* and *Cnn1*. For the remaining experiments, 2.5 μg/ml Jagged1 was used as this concentration

was sufficient to show an induced contractile SMC expression and optimizes loss of protein in the wash fraction.



Immobilization Efficiency				
Jagged1 Concentration	Absorbance	Bead Volume (µL)	Total Protein in Wash Fraction (µg)	Total Protein Immobilized (µg)
Jagged 0	0	20	0	0
Jagged 2.5	0.0055	20	0.174	2.326
Jagged 5	0.0405	20	1.285	3.715

Figure 4-3 Jagged1 immobilization to Protein G beads in a concentration-dependent manner. Recombinant Jagged1 was immobilized to Protein G magnetic Dynabeads™ through absorption for 10 mins on rotation at room temperature. **A)** Wash fractions were obtained and quantified by an ELISA assay then subtracted from the total protein immobilized to quantify total protein immobilized to the bead fraction **B).** Immobilized Jagged1 beads with 0-5 µg pre-coat were culture with HCASMCs and gene expression levels of A*cta2* and *Cnn1* were investigated using RT-qPCR. * indicates significance from the control $p<0.05$.

4.1.1.4. Immobilized Jagged1 directs protein levels of HCASMCs

The effect of immobilized Jagged1 driven SMC response was also translated to the protein level evaluated both by Western blot (**Fig. 4-4)** and immunofluorescence microscopy **(Fig. 4-5)**. Both immunofluorescence microscopy and Western blot analysis demonstrated that soluble Jagged1 had no effect on the SMC contractile genes. Additionally, soluble Jagged1 did not influence Jagged1 ligand or Notch3 receptor protein levels (**Figure 4-4 B, C**).

Similar to the gene expression data for immobilized Jagged1, protein analysis also demonstrated a positive role of immobilized Jagged1.The upregulation of Acta2 was shown using Western Blot (**Figure 4-4 D),** demonstrating a 3-fold increase in relative band intensity, and immunofluorescent imaging (**Figure 4-5 D)** of Acta2 revealed a 2.5-fold increase in relative fluorescence intensity when normalized to the control. Furthermore, Cnn1 (**Fig. 4-4 E**) showed an

upregulation, in line with the gene expression data presented previously. Lastly, an unchanged Myh11 immunofluorescence expression indicated that Jagged1 beads did not have an observable effect (**Fig. 4-5 F**). Collectively, these data strongly indicate the influence of immobilized Jagged1 on early-stage contractile protein markers was more considerable than the late-stage markers.

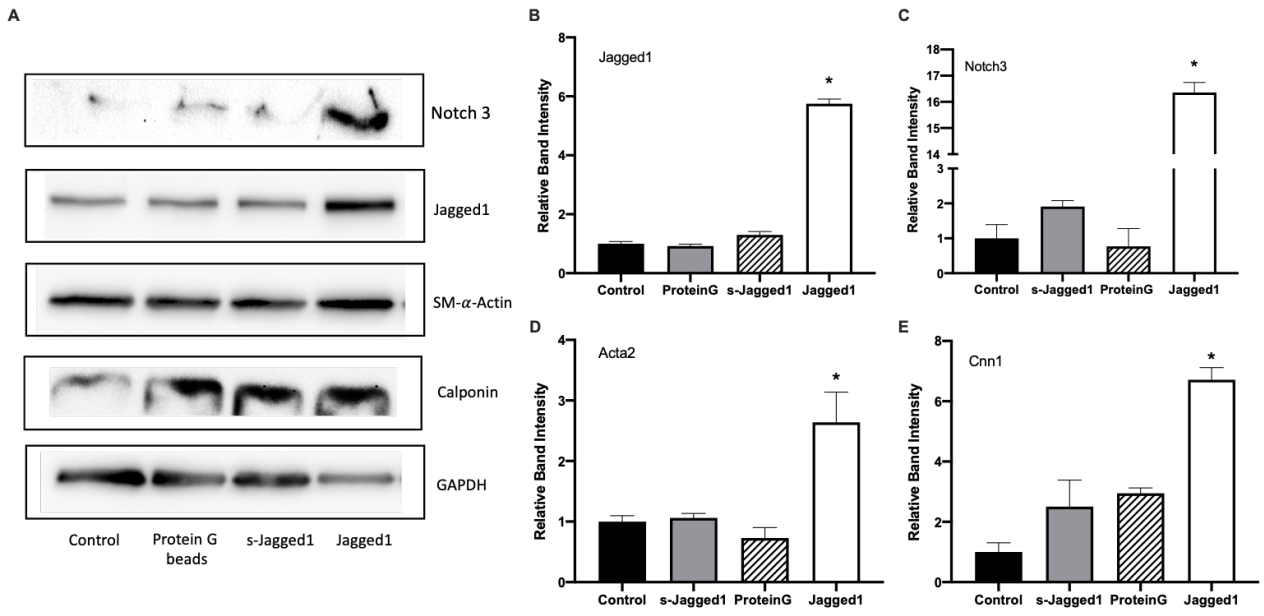


Figure 4-4 Western blot analysis of Notch induced contractile proteins Jagged1 and Notch3 protein levels.
Jagged1 driven SMC contractile marker protein expression was analyzed using Western Blot analysis. Representative blots are shown in **A)**. Western Blot band intensities of **B)**Jagged1, **C)**Notch3, **D)**Acta2, and **E)**Cnn1 were quantified using ImageJ. Band intensities were normalized to GAPDH and plotted as the normalized expression of the untreated culture. Three independent band readings were taken, and the * indicates significance ($p<0.05$).

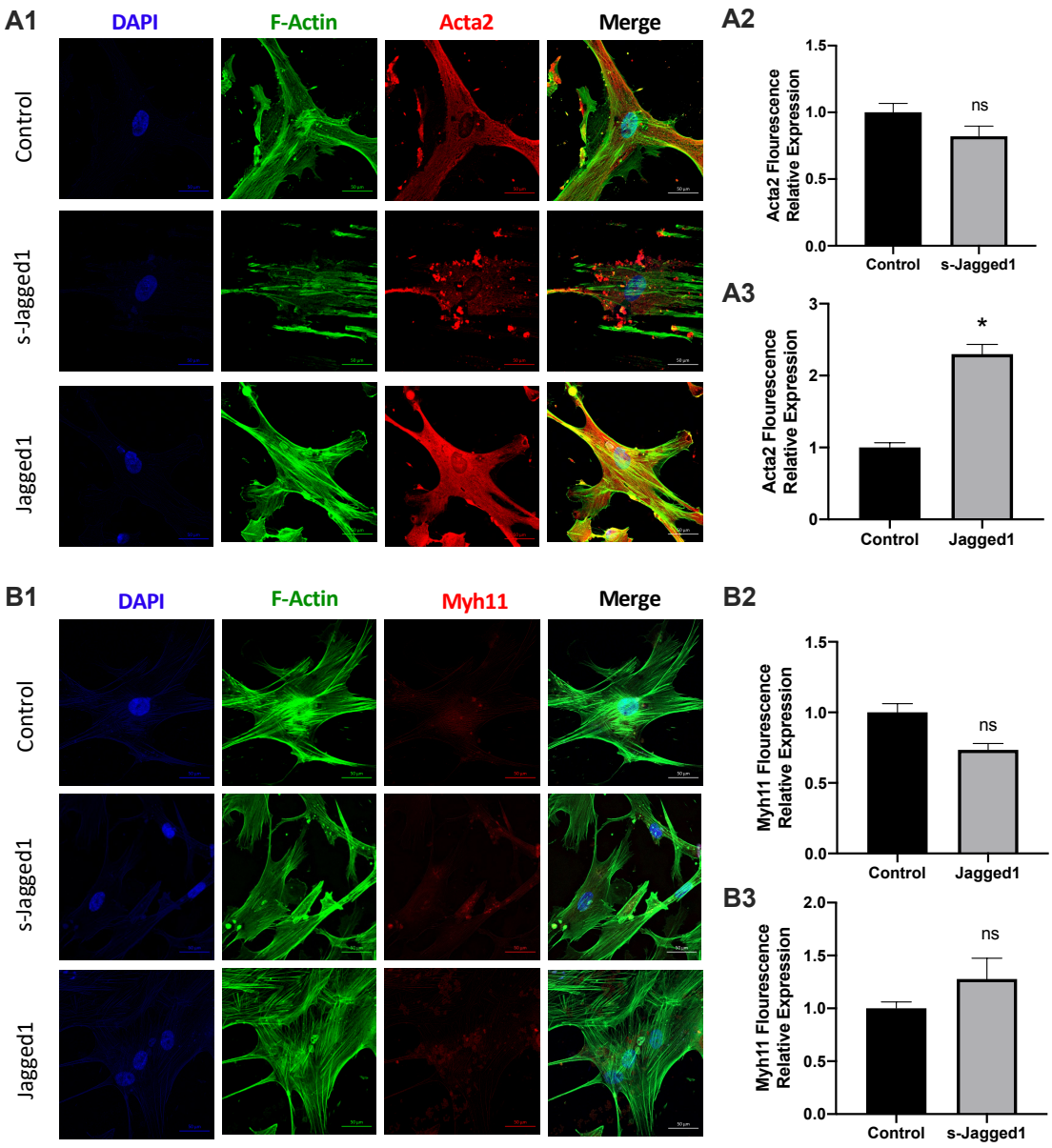


Figure 4-5 Contractile marker protein expression of HCASMC's with the treatment of soluble and immobilized Jagged1.
Immunofluorescence staining of HCASMCs comparing soluble and immobilized delivery of Jagged1 were used to determine the expression of contractile protein markers upon Notch activation. Cells were stained with **A1)** early-stage contractile marker Acta2 and **B1)** late-stage contractile marker Myh11. In all panels moving horizontally to the right show DAPI staining (blue), F-Actin staining (green), the protein of interest (red) and merged images. Scale bar: 50 μm. HCASMCS cultured alone were used as a control. Quantification (using ImageJ) of the relative fluorescence quantification of Acta2 expression **A2, A3),** and Myh11 expression **B2, B3)** are shown to the right. Data are presented as the mean fluorescence from multiple readings ± SD normalized to the control HCASMCs cultured alone in SmGM. * indicates statistical significance in comparison to the control group at $p<0.05$.

The representative Western blots also demonstrate the influence of Jagged1 immobilization on relative ligand-receptor (Jagged1-Notch3) expression levels in HCASMCs (**Fig. 4-4 B,C).** These results suggest that immobilized Jagged1 also had control of the relative ligand-receptor expression levels. Ligand and receptor levels are important because Notch is driven by stochiometric interactions compared to other signaling pathways which are enzymatically driven. Thus, the Notch target gene expression is reported to increase in a dose-dependent manner[34–36]. Bead-bound Jagged1 delivery were able to enhance the expression of Notch3 expression and subsequently Jagged1 expression in the signal-receiving cell shown by the Western blot (quantified in **Fig 4-4 B**). The induction of Jagged1 expression in the differentiated SMCs is critical to support a simple feed-forward pathway by sequentially activating differentiation and maintaining homeostasis in the subsequent SMC layers, a process known as lateral induction[37].

4.1.2. The effect of Notch inhibitor on HES1 and contractile marker expressions

Since evidence has shown and demonstrated a critical role of Notch signaling in both vascular remodeling as well as pathogenesis and disease in cardiovascular health, both activation and inhibition of Notch signaling can be important for designing treatments. The use of gamma-secretase inhibitors, including DAPT have been shown to inhibit or attenuate the Notch signaling pathway in literature [20,39–41]. To directly implicate Notch to SMC contractile phenotype, a Notch-specific gamma-secretase inhibitor DAPT was used to attenuate Jagged1-induced Notch signaling shown in **Figure 4-6**. Mechanistically, DAPT blocks the Notch intracellular domain (NICD) cleavage at the S3 domain, which prevents subsequent translocation of Notch3 to the nucleus (**Fig. 4-6 A**). DAPT treatment of HCASMC attenuated both the Notch transcription factor *HES1* and contractile protein markers *Acta2* and *Cnn1* (**Fig. 4-6 B-D**). However, there was no significant

effect on *Myh11* (**Fig. 4-6 E**)*;* therefore, Notch may not be directly responsible for *Myh11* expression levels and may rely on the interaction with other signaling pathways.

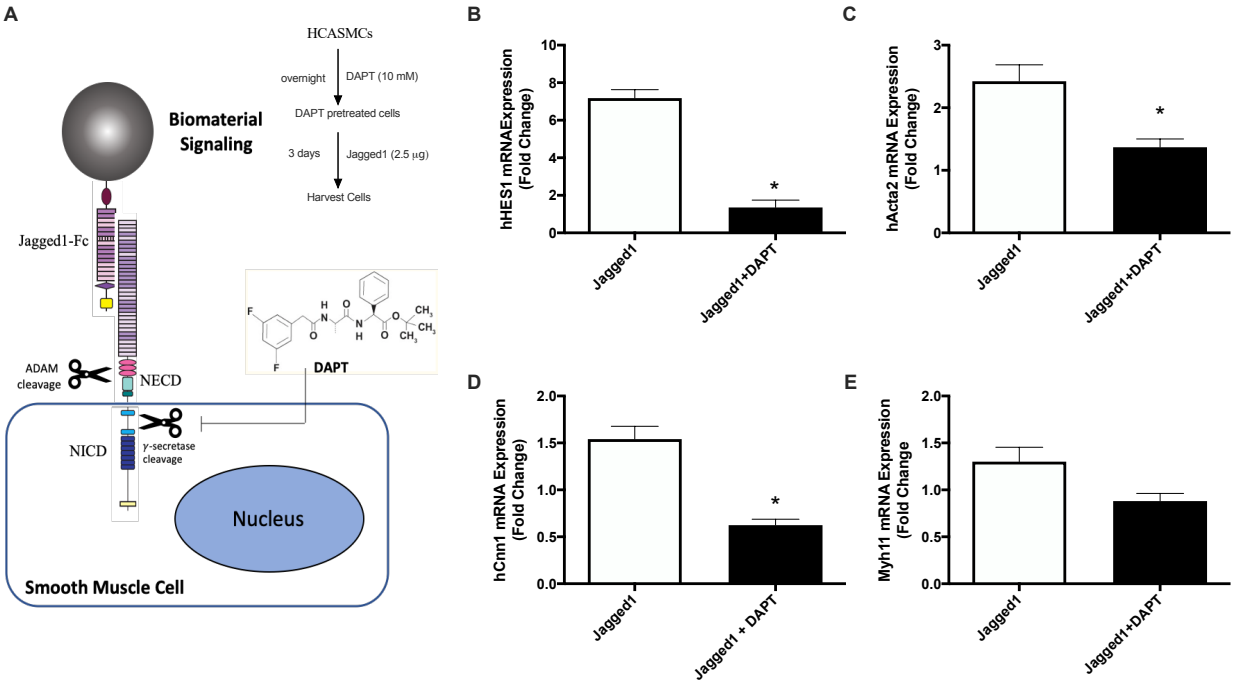


Figure 4-6 Smooth muscle cell phenotype was directly affected by activating Notch signaling, demonstrated by a specific Notch inhibitor DAPT.
HCASMCs were cultured on a 24 well plate for 2 days. On day 2, cells in the DAPT group were treated overnight with 10 μM DAPT. On day 3, SMC media was exchanged and then cells were incubated for 3 days with i) immobilized Jagged1/Fc beads or ii) DAPT (10μM) and Jagged1-Fc immobilized beads. DAPT, a specific S3 inhibitor, preventing cleavage of the NICD **A).** Expression levels of ***B)*** *HES1*, **C)** *Acta2*, **D)** *Cnn1*, and **E)** *Myh11* were quantified by RT-qPCR and normalized to the untreated HCASMC culture control. Significant downregulation by DAPT inhibitor suggests a clear link between Notch signaling and these target proteins, demonstrating Notch signaling cause and effect in HCASMCs. Treatment groups were normalized to the SMC control set to a value of 1 (not shown) and represented as the normalized fold change. * indicate statistical significance in comparison to the control group at $p<0.05$.

4.1.3. Serum starvation of HCASMC for enhanced contractile protein expression

It was surprising that HCASMCs did not express mature contractile markers, including myosin heavy chain in the previous experiments. Myosin heavy chain is a hallmark contractile protein for mature smooth muscle cells. In culture HCASMCs generally undergo a phenotype switch and acquire a more synthetic phenotype. To test the capability of these cells to undergo phenotype transition and express mature markers (which was not seen with immobilized Jagged1), other factors and culture conditions were investigated to drive this transition. Serum deprivation is a known culture condition to induce a phenotypic change of vascular smooth muscle cells with an elongated/spindle-shaped morphology, an elevated myofilament density, and reacquired contraction[38]. Therefore, this was used to induce functional protein expression and a contractile morphology. To analyze this response and stimulate late-stage contractile markers, HCASMCs grown to sub-confluence were serum-starved for 72 hours and imaged using immunofluorescent microscopy (**Fig. 4-7).** The addition of 2ng/mL TGFβ1 was also used as an additional factor to induce further functional protein expression and phenotype switching.

Serum starvation successfully caused morphological changes in the HCASMCs. Cells were morphologically elongated and spindle-shaped upon serum starvation which is indicative of a mature contractile SMC. Serum starvation of HCASMCs was also able to significantly stimulate the expression of Calponin1/2/3, similar to expression levels as seen with immobilized Jagged1 (**Fig. 4-7 A**). Expression of myosin heavy chain and smoothelin were induced with these treatment conditions which were lacking upon Jagged1 treatment (**Fig. 4-7 B,C)**. This increase was also enhanced upon treatment with TGFβ1 in the culture media. Therefore, serum starvation, in combination with TGFβ1 enhanced transition into a mature contractile phenotype. This also

suggests that bead-bound Jagged1 alone may not be an optimal culture system to induce a fully mature contractile SMC.

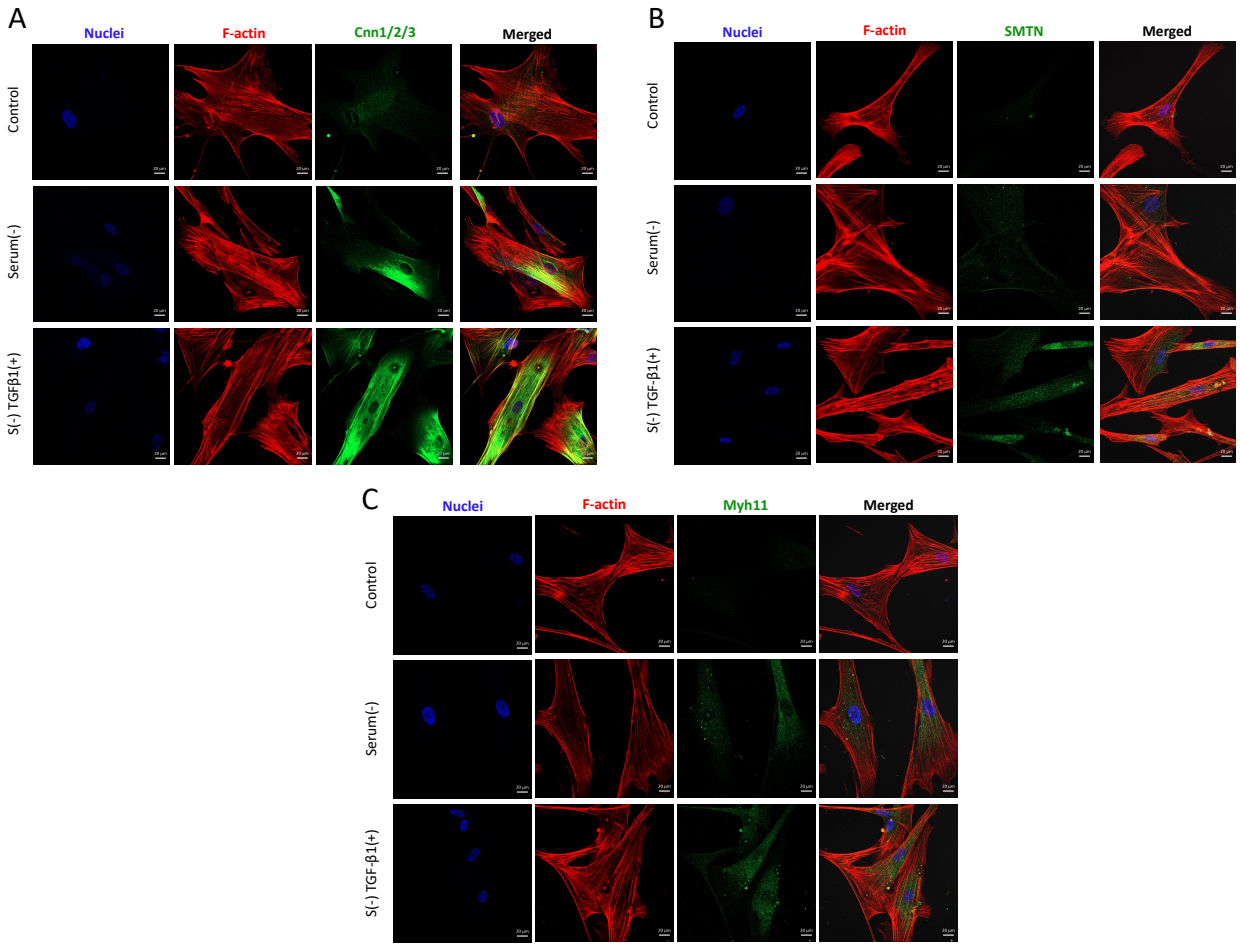


Figure 4-7 Serum starved HCASMC protein expression and morphological changes
HCASMCs were cultured for 72 hours in serum-free-DMEM, with and without 2ng/mL TGFβ1 to further induce contractibility. HCASMCS cultured alone in SmGM were used as a control. Immunofluorescent staining of **A)** Cnn1/2/3, **B)** Myh11 and **C)** Smtn is shown. Scale bar: 20 μm.

4.1.4. Jagged1 signaling cross-talk with TGFβ1 in HCASMC

Many developmental processes that are regulated by Notch signaling are also controlled by TGFβ family of ligands, including BMP and TGFβ. As discussed in **Section 2.7.1,** Notch signaling has been identified to play a role in the vasculature and ligands such as TGFβ1 have been

identified for providing a specific role in SMC phenotype control[2], ECM synthesis, VSMC proliferation and VSMC migration[42]. The influence of both Jagged1 and TGFβ1 on the expression of smooth muscle cell markers are presented in **Figure 4-8**.

The data demonstrated that both Jagged1 and TGFβ1 direct SMC regulation by significant upregulation of *HES1* a Notch transcription factor as well as contractile protein markers *Acta2* and *Cnn1*. Jagged1 signaling influenced the expression of *HES1* more drastically as there was an 8-fold increase compared with 3.5-fold increase with TGFβ1 (**Figure 4-8 A)**. It is interesting that TGFβ1 also influenced *HES1* which is not a direct target, but this upregulation of HES1 is consistent with other reports in literature[43]. TGFβ1 is a more prominent effector on SMC response, especially with late-stage contractile genes (**Figure 4-8 B,C**). Using TGFβ1 treatment alone, it was possible to significantly upregulate *Myh11* expression which was not achieved with Jagged1 Notch treatment. A limitation of this study was that the relative concentration dependence was not investigated and is an avenue for future research.

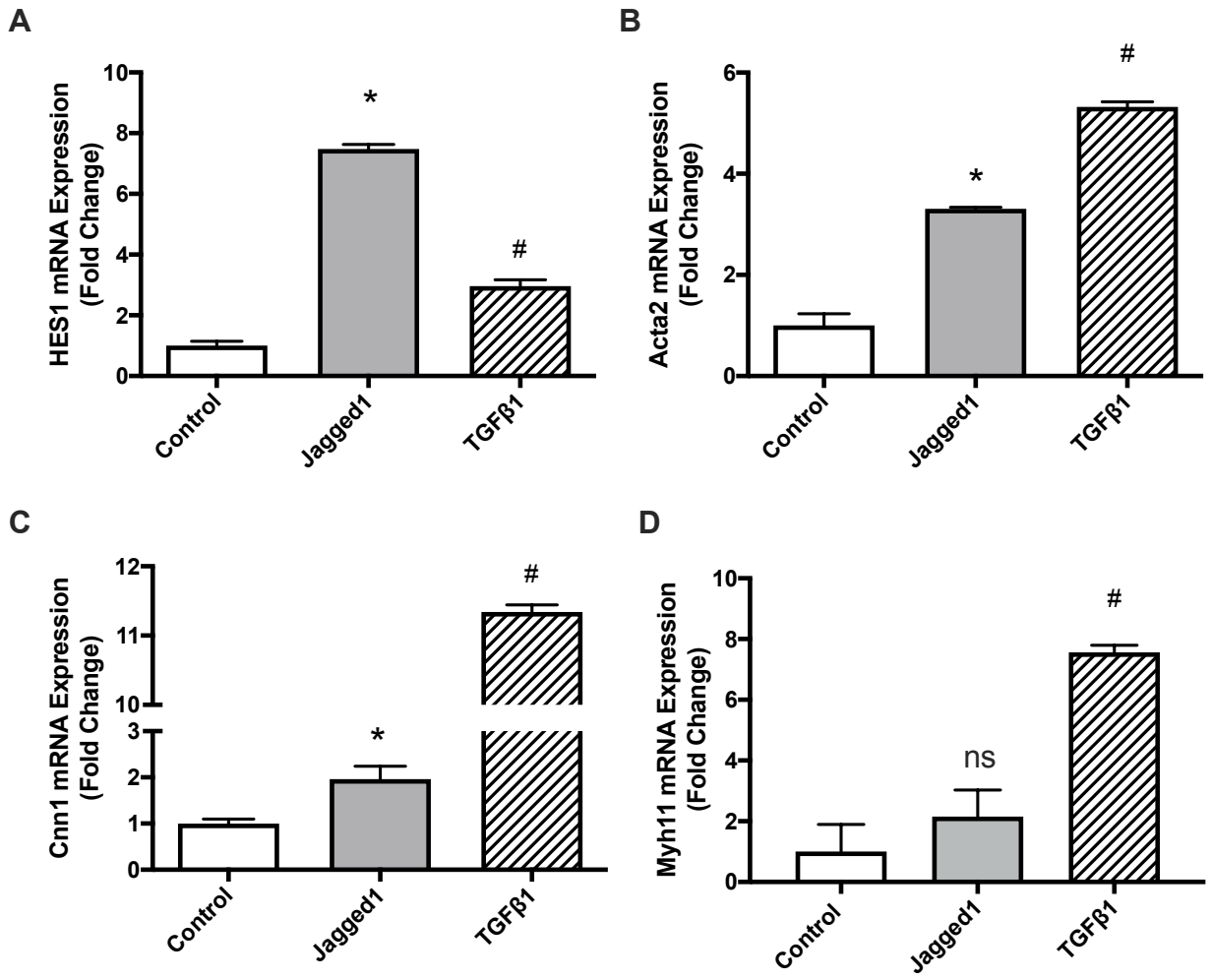


Figure 4-8 Comparison of Jagged1 and TGFβ1 ligands to direct HCASMC phenotype control.
HCASMCs were cultured for 3 days in a 24-well plate with the addition of i) immobilized Jagged1/Fc beads (2.5μg/mL) or ii) 2ng/mL soluble TGFβ1. Expression levels of **A)** *HES1*, **B)** *Acta2*, **C)** *Cnn1*, and **D)** *Myh11* were quantified by RT-qPCR and normalized to the untreated HCASMC culture control. Significant upregulation of all genes was observed with both TGFβ1 treatment. Jagged1 signaling in comparison only upregulated *HES1, Acta2* and *Cnn1*. Both of these pathways thus play a direct role in SMC control and differentiation. Data is presented as the mean ± SD normalized to the control HCASMCs cultured alone in SmGM, and the symbols (*, #) indicate significance from each other $p < 0.05$.

Since Jagged1 and TGFβ1 ligands both contribute to vascular development and pathogenesis[44–47], the integration of these two pathways could be useful[29]. The ability for Notch signaling components to interact, impact, and cross-talk with multiple signaling

pathways/components adds complexity to controlling Notch signaling. Although the current understanding of the molecular mechanisms involved in Notch cross-talk is still in development, there is evidence that several pathways are interconnected. The integration of multiple pathways within the body is suggested to play a role in the abundance of Notch ligands and receptors before receptor binding, and there is convergence within pathways in the body (discussed in **Table 2-4, Section 2.7.1**). Notch and TGFβ signaling have been identified as co-regulators of Smad proteins indicating cross-talk between these pathways[48,49],[50]. In **Section 2.7.1** a discussion was provided about the direct protein interaction within the two signaling pathways where the NICD cooperatively interactions with Smad2/3[2], an intracellular transducer of the TGFβ signaling pathway.

In this study, the role of bead-bound Jagged1 and TGFβ1 was investigated in combination. In these experiments, HCASMCS were cultured for 3 days with immobilized Jagged1 beads (2.5μg/mL, 200 beads/cell), soluble TGFβ1(2ng/mL), or combination treatment. Results shown in **Figure 4-9 A** indicated that both Jagged1 and TGFβ1 directly upregulate *HES1* gene expression significantly compared to the controls in which HCASMCs were cultured in SmGM. In the combination treatment, *HES1* expression is also slightly increased compared with Jagged1 or TGFβ1 treatment group. Therefore, there is an added effect of Notch activation by using a combination of the two ligands. When analyzing the effect of Jagged1 and TGFβ1 treatment on smooth muscle cell contractile genes, both treatment groups significantly upregulated SMC contractile genes *Acta2*, and *Cnn1* as shown in **Figure 4-9 B,C**. Again, the combination treatment significantly upregulated these contractile markers further. Lastly, TGFβ1 treatment significantly upregulated *Myh11* expression which then was increased slightly by the combination treatment of

Jagged1 and TGFβ1 (**Figure 4-9 D)**. It is interesting to note that alone Jagged1 has no effect on *Myh11* expression but provided added therapeutic benefit in combination with another signaling ligand.

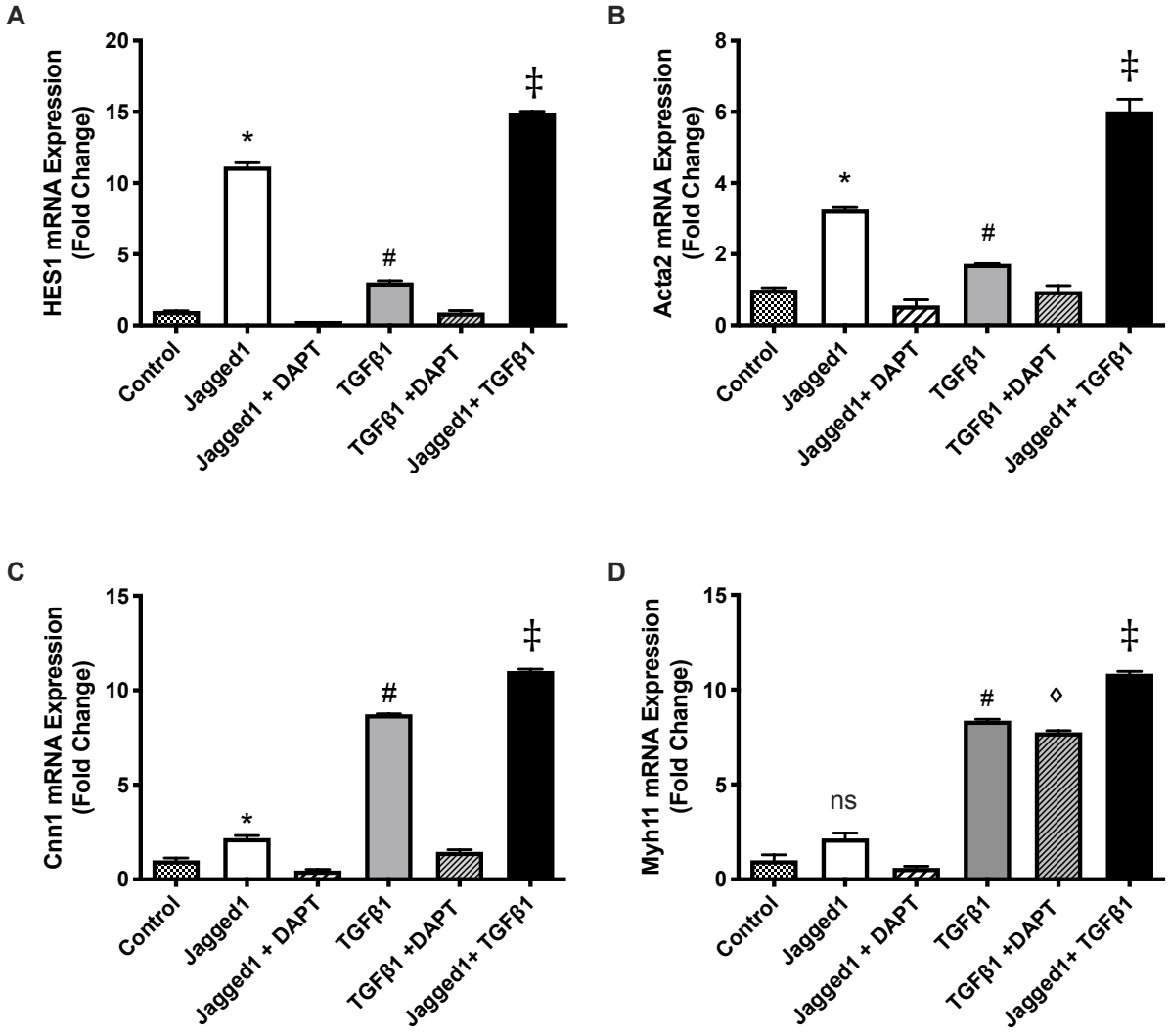


Figure 4-9 Investigating the synergistic relationship between TGFβ1 and Jagged1 ligands. HCASMCs were cultured for 3 days with immobilized Jagged1 beads (2.5μg/mL, 200 beads/cell), soluble TGFβ1 (2ng/mL) or a combination treatment. DAPT, a Notch specific inhibitor, was also added in combination to demonstrate Notch-specific response of both ligands. Gene expression levels of human *HES1* **(A),** *Acta2* **(B),** *Cnn1* **(C),** and *Myh1*1 **(D)** are shown. The symbols indicate statistically significant. Data are presented as the mean ± SD normalized to the control HCASMCs cultured alone in SmGM. Symbols indicate significance compared to the control $p < 0.05$. Control refers to HCASMCs cultured in SmGM

Cross-talk was further confirmed using a Notch inhibitor. DAPT inhibits the (S3) intracellular domain cleavage of the Notch receptor. Since TGFβ1 signaling is also attenuated by DAPT, there may be a relationship between the NICD and TGFβ intracellular components. DAPT specifically attenuated signaling in both Jagged1 and TGFβ1 treated cells for *HES1, Acta2,* and *Cnn1*. It is important to note that *Myh11* gene expression was not significantly attenuated by DAPT when treated with TGFβ1, suggesting mechanisms other than Notch signaling may be in play for this gene. Since Jagged1 and TGFβ1 concentrations were fixed, it is unknown if dosing has an effect. The relative contribution of each ligand in the combination treatment was not studied.

In addition to RT-qPCR data, immunofluorescence and Western blotting were used to evaluate protein levels. As shown in **Figure 4-10 A-D,** calponin which is one of the early-stage SMC differentiation markers, was robustly expressed at the protein level by both TGFβ1 and Jagged1. Consistent with the RT-qPCR data, the Notch inhibitor attenuated calponin. Contrary to calponin, the late-stage contractile marker smoothelin was not affected by either TGFβ1 or Jagged1 (**Figure 4-10 E**). The autofluorescence of protein G beads can be seen in the red channel and was discounted. Although a fully matured HCASMC is known to express smoothelin, the absence of this marker suggested the lack of maturity – a hallmark for the synthetic phenotype. Longer duration in a Jagged1 activated state might also be required to show significant smoothelin expression.

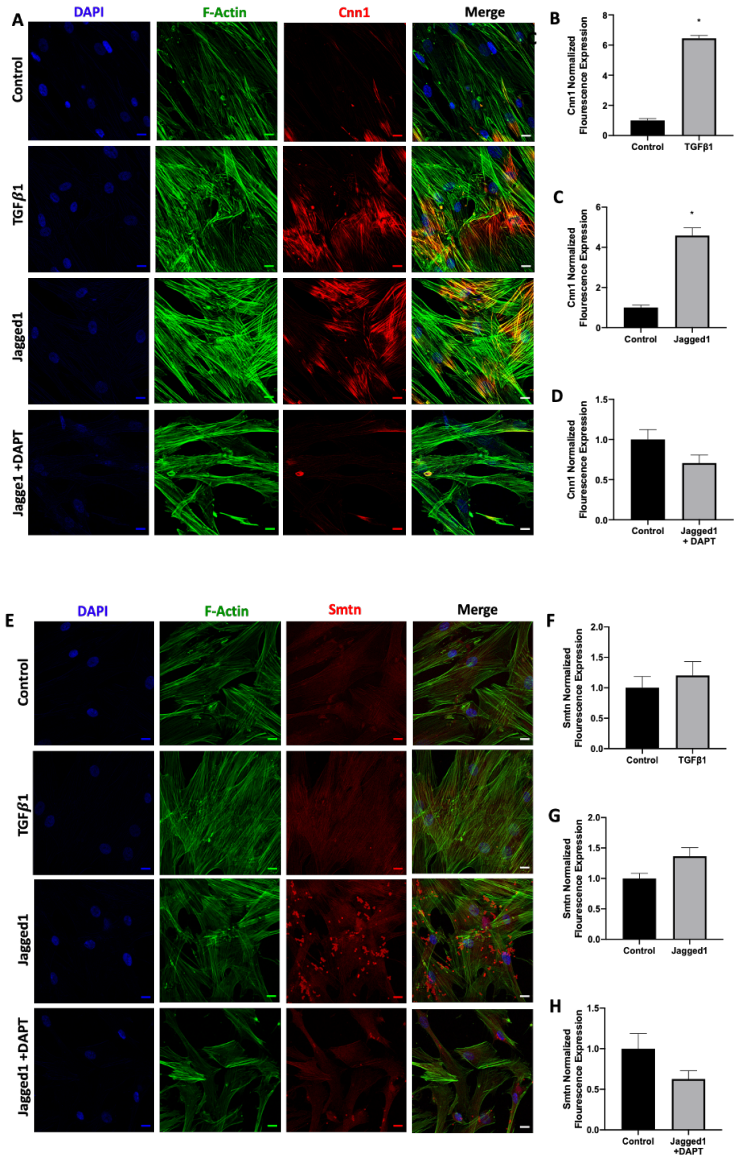


Figure 4-10 Effect of Jagged1 and TGFβ1 on protein expression in HCASMCS to control smooth muscle cell markers analyzed by immunofluorescence imaging

HCASMCs were cultured for 2 days, and then incubated for an additional 3 days with the following treatments i) Jagged1-Fc (2.5µg/mL) immobilized beads (200 beads/cell), ii) Jagged1-Fc immobilized beads (2.5µg/mL) + DAPT (10µM), and iii) TGβ1 (2ng/mL). Protein level was confirmed by immunofluorescence staining. Calponin (Cnn1) **(A)** and smoothelin **(E)**. The bar graphs are the corresponding quantification of the images; **(B-D)** for calponin and **(F-G)** for smoothelin. Scale bar= 50μm. Both Jagged1 and TGFβ1 significantly showed increased expression of Cnn1 but not Smtn. Data is presented as the mean fluorescence intensity from multiple readings ± SD normalized to the untreated control HCASMC. * indicates statistical significance in comparison to the control group at $p<0.05$.

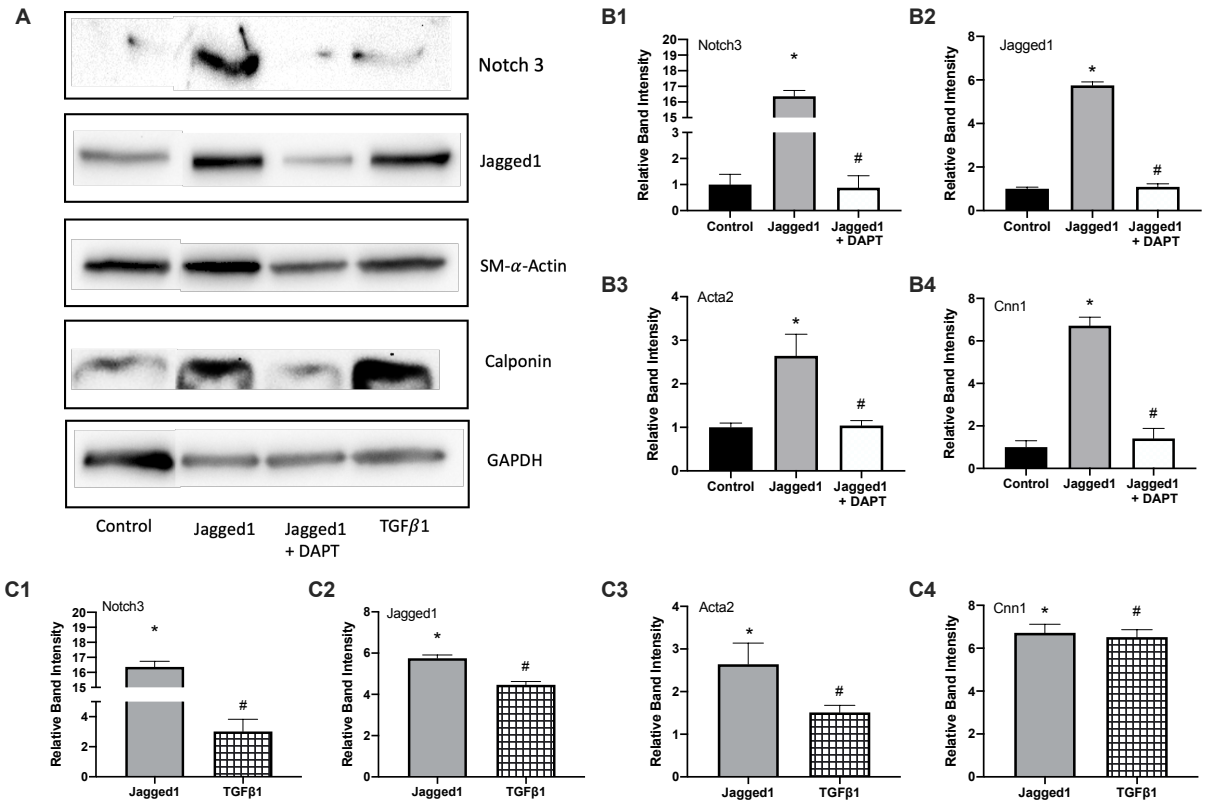


Figure 4-11 Effect of Jagged1 and TGFβ1 on protein expression in HCASMC to control smooth muscle cell markers analyzed by Western blot

HCASMCs were cultured for 2 days, and then incubated for an additional 3 days with the following treatments i) Jagged1-Fc (2.5μg/mL) immobilized beads (200 beads/cell), ii) Jagged1-Fc immobilized beads (2.5μg/mL) + DAPT (10mM), and iii) TGβ1 (2ng/mL). Protein expression was confirmed by Western Blot analysis, and representative blots of the experiment are shown in **A).** Band intensities were normalized to GAPDH and plotted as the normalized expression of the untreated control culture. HCASMCs cultured in SmGM served as controls. The relative band intensities for Jagged1 and DAPT treated cells are quantified in **B1-B4**. The relative band intensities for Jagged1 *vs.* TGFβ1 ligand presentation were quantified in **C1-C4**. Data are represented as mean band reading ± SD. The symbols (*,#) indicate statistically significant differences from each other ($p<0.05$).

Western blot was also used to confirm the gene expression data (**Fig 4-11 A**). An upregulation of Notch3 (~16-fold), Jagged1 (~6-fold), Acta2 (~3 fold), and Cnn1 (~ 7-fold) was observed and was attenuated by DAPT treatment. The data also showed that both Jagged1 and TGFβ1 are able to upregulate the expression of both Acta2 and Cnn1 protein expression. To summarize the role of Jagged1 on HCASMC, immobilization was a necessary, perhaps not a sufficient condition for

enhancing phenotype modulation and revealed evidence for cross-talk between Notch and TGFβ intracellular components.

4.2. Effects of Jagged1 delivery for vascular smooth muscle cell differentiation of stem and progenitor cells

HCASMCs are a great model to investigate Jagged1-specific Notch activation and response. Nevertheless, HCASMCs can only be used as a model cell since harvesting them from the coronary arteries of patients is not feasible. Therefore, other model cells, and autologous cell sources need to be explored. Cellular therapies often use allogenic or autologous sources for therapeutic strategies; however, it is often difficult to maintain a homogenous and convenient cell source to generate cells with a stable phenotype and function. In this study iPSC-MSCs and 10T1/2 cells were investigated for their Jagged1-specific differentiation.

4.2.1. The effect of Jagged1 on iPSC-MSC differentiation towards VSMC

Rather than using primary cell sources, an alternative cell source for vascular tissue engineering is induced pluripotent stem cells (iPSC)[51]. iPSC-MSCs can be derived from human skin fibroblast cells and can become reprogrammed by viral overexpression of specific transcription factors. Retroviral transduction of Oct4, Sox2, Klf4, and c-myc is a common approach[52,53]. These iPSCs can then be differentiated into an MSC lineage and further matured into a smooth muscle cell. This process is summarized in **Figure 4-12.**

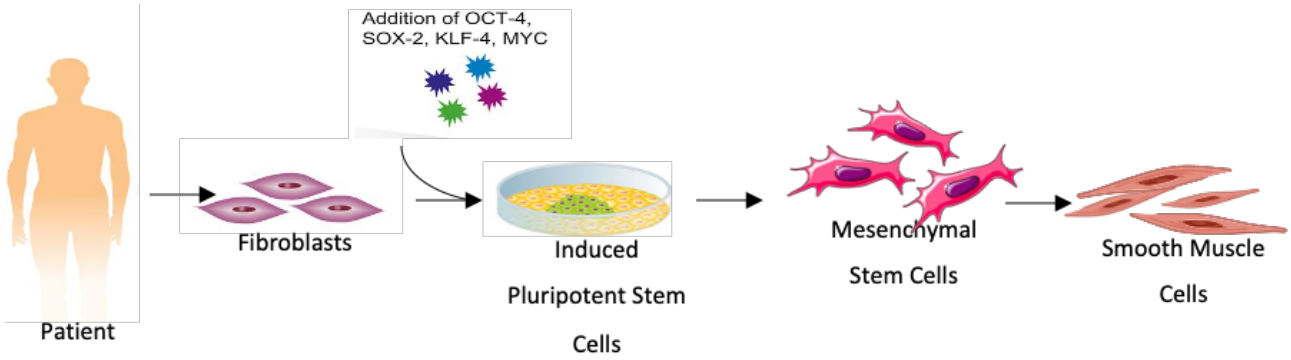


Figure 4-12 Generation of patient specific iPSCs from fibroblast cell reprogramming and subsequent differentiation to a smooth muscle cell lineage.

Notch signaling has been a target for iPSC-MSC cell fate decisions into cardiac and neuronal differentiation. For example, ascorbic acid for mesoderm induction, followed by DAPT (Notch inhibition) accelerated the generation of beating cardiomyocytes[54]. Compared to bone marrow-derived MSCs, vascular differentiation of iPSC-MSCs were less responsive to traditional differentiation protocols and thus proved to be difficult[55]. Since Notch signaling is critical in development and specifically cell fate determinations, including progenitor differentiation, it is beneficial to determine if Notch signaling could be harnessed for mature contractile SMC differentiation. The induction of smooth muscle gene expression in mesenchymal stem cells has been effective in activating Notch signaling as demonstrated by HES1 upregulation, and increased Myh expression[56]. Therefore, the aim here was to determine if Jagged1 could direct iPSC-MSC differentiation into a smooth muscle cell lineage.

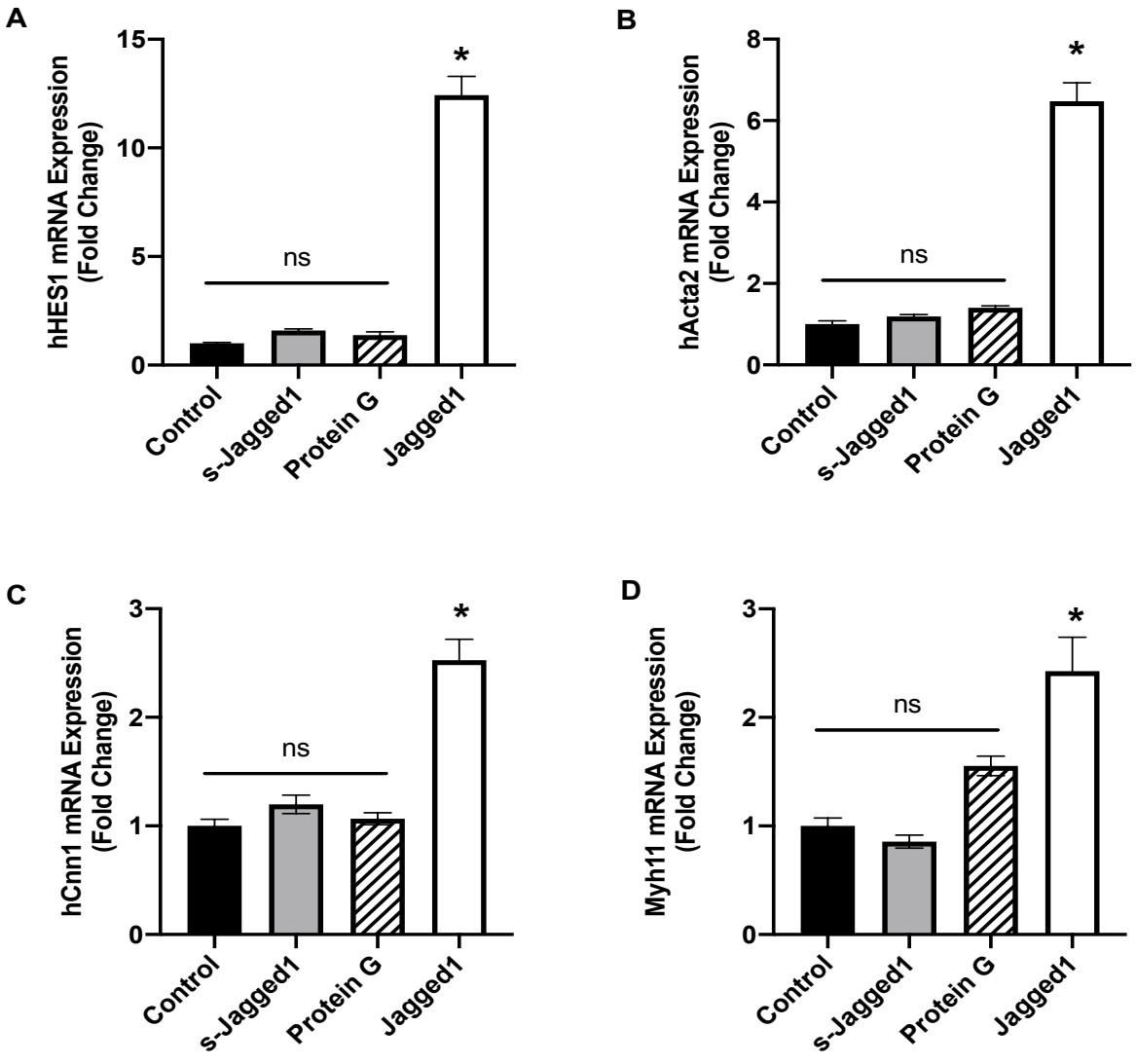


Figure 4-13 Differentiation of iPSC derived MSCs by Jagged1
iPSC derived mesenchymal stem cells were cultured for 4 days followed by the addition of soluble Jagged1, protein G beads or Jagged1 immobilized beads for an additional 3-day culture. RT-qPCR analysis revealed that soluble Jagged1 has no effect, but immobilized Jagged1 significantly upregulates **A)** *HES1* transcriptional factor and smooth muscle cell (SMC) contractile marker genes (**B)** *Acta2, **C)** Cnn1* and **D)** *Myh11*). The bead concentration was 200 beads/cell and 2.5 µg of Jagged1 protein was immobilized or added as a soluble protein. Data are represented as mean ± SD normalized to the control HCASMCs cultured alone in SmGM. * indicates significance at $p < 0.05$.

As was seen previously for HCASMCs, iPSC-MSCs were also responsive to Jagged1(**Figure 4-13)**. While soluble Jagged1 did not influence differentiation, immobilized Jagged1 was able to differentiate iPSC-MSCs into a SMC lineage. Both an upregulation of the Notch transcription factor *HES1* and contractile protein markers smooth muscle *Acta2* and *Cnn1* and *Myh11.* This was linked directly to the Notch signaling pathway because DAPT was able to attenuate iPSC-MSC differentiation.

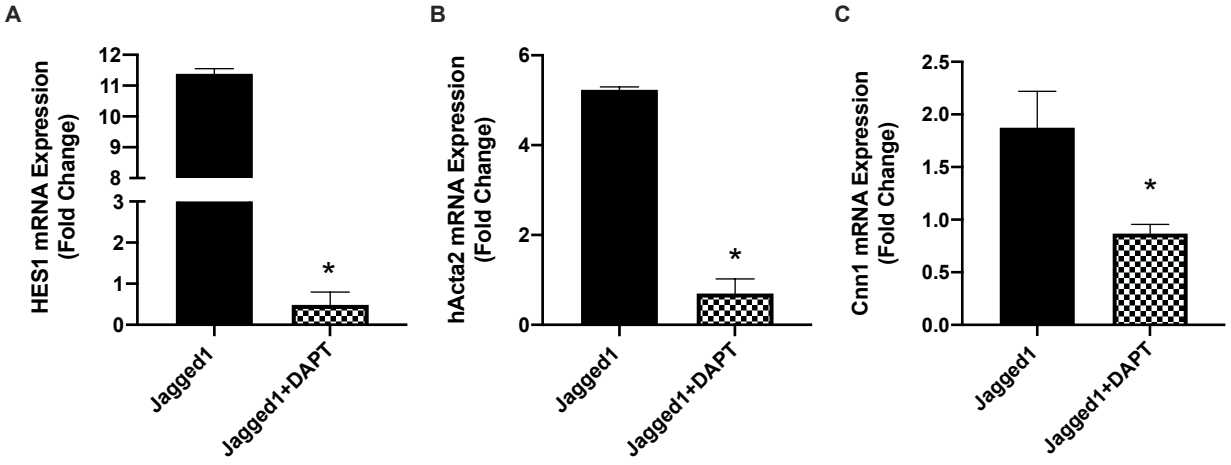


Figure 4-14 Vascular differentiation of iPSC-MSC using immobilized Jagged1
iPSC-derived mesenchymal stem cells were cultured for 4 days, then of Jagged1 immobilized beads was added for an additional 3-day culture. The addition of 10 μM DAPT pre-treatment overnight followed by a combination of 2.5μg/mL Jagged1 + 10 μM DAPT. Expression of **A**) *HES1* **B**) *Acta2*, and **C**) *Cnn1*. Data are presented as the mean ± SD normalized to iPSC-MSC's cultured alone in SmGM (not shown). * indicates significance compared to Jagged1 treated iPSC-MSCs, $p < 0.05$.

Similar effects were seen using Western blot analysis (**Figure 4-15).** These data suggested that undifferentiated iPSC cells could be driven towards a SMC lineage and could be useful for vascular tissue engineering. Quantification of the representative blots shown in **Figure 4.15** indicates the increased expression of HES1 but not Myh11 at the protein level. These cells showed a similar response to HCASMCs. Jagged1 beads also increased the expression of Acta2 and Cnn1. DAPT was able to attenuate the Jagged1 expression, supporting the previous observation. In conclusion,

immobilized Jagged1 could be useful in directing the differentiation of iPSC-MSC towards a smooth muscle cell lineage. These cells still lack the expression of more mature contractile markers, including Myh11. It is suspected that other biochemical factors, along with Jagged1 may be required to fully mature these cells.

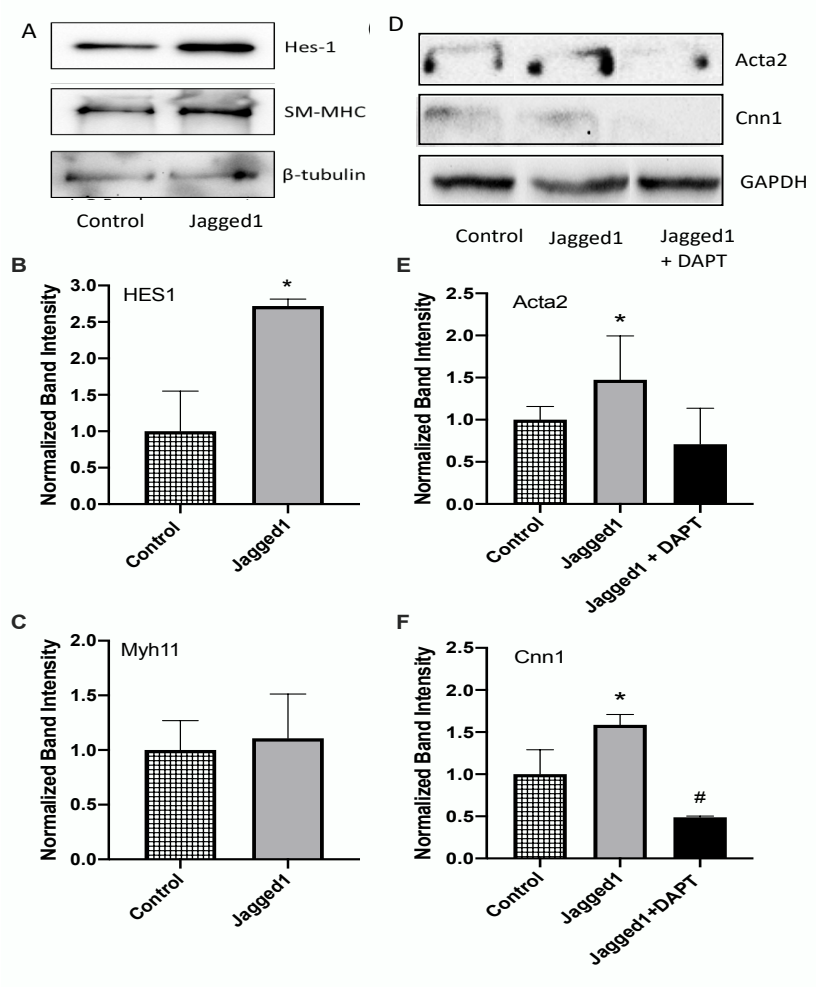


Figure 4-15 Smooth muscle protein expression of iPSC-MSCs driven by Jagged1 signaling
iPSC derived mesenchymal stem cells were cultured for 4 days and then treated with Jagged1 beads (200 beads/cell and 2.5μg/mL of Jagged1). Western Blot analysis was done to confirm protein expression. Representative blots are shown in **A)**. The relative band intensity for **B)** HES1, and **C)** Myh11. iPSC-MSCs cultured alone served as controls. To confirm a Notch specific differentiation, representative blots are shown in **D)**. Relative band intensity of both **E)** Acta2 and **F)** Cnn1 were quantified. Data are represented as mean blot intensity ± SD. Treatment groups were normalized to the iPSC-MSC controls grown in SmGM and represented as the normalized fold change. The symbols (*,#) indicate statistically significant differences from each other ($p<0.05$).

4.2.2. Vascular differentiation of 10T1/2 cells using Jagged1

Extending the findings of HCASMC and iPSC-MSC, the use of bead-bound Jagged1 was expanded to the mouse embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells). These cells have been used as a model for cartilage and bone tissue engineering and gained attention in vascular tissue engineering[57]. The differentiation of 10T1/2 cells into a SMC lineage has been demonstrated using a co-culture with endothelial cells or treatment with TGFβ1[58,59]. In this thesis, the aim was to determine the influence of Jagged1 delivery by studying the effects of TGFβ1 and Jagged1 on 10T1/2 cells differentiation. In the first experiment the potential of these cells to differentiate and express smooth muscle cell markers in response to 2ng/mL TGFβ1 **Figure 4-16** was observed**.**

Upon delivery of TGFβ1, *HES1***,** *Notch3*, and SMC markers *Acta2 ,Cnn1,*and *Myh11* are significantly upregulated. A similar protein expression of Acta2, Smtn and Myh11 to that of HCASMCs was seen with the treatment of TGFβ1 which has been recently published[58]. This differentiation approach using TGFβ1 is attractive; however, research has shown that TGFβ1 failed to fully differentiate these into SMCs due to inconsistent expressions of myocardin, smoothelin, and myosin-heavy chain[60–62].

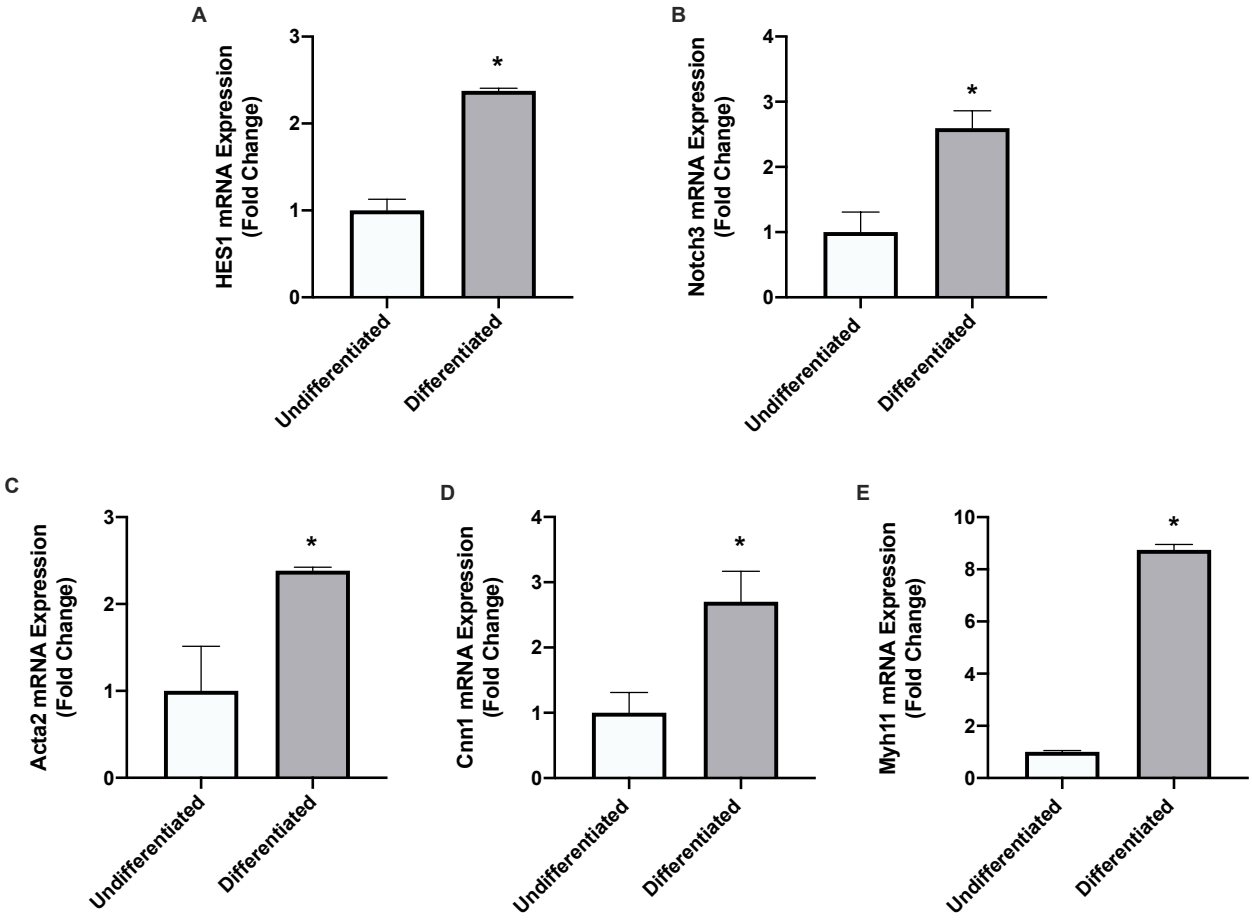


Figure 4-16 Differentiation of embryonic multipotent mesenchymal progenitor cell (10T1/2 cells) into a smooth muscle cell lineage using TGFβ1
10T1/2 cells were cultured for 3 days in DMEM with 2ng/mL TGFβ1. RT-qPCR analysis of **A)** *HES1, **B)** Notch3* and contractile SMC markers, **C)** *Acta2*, **D)** *Cnn1* and **E)** *Myh11* were used to show differentiation of 10T1/2 cells into a SMC lineage. Upregulation of all three markers demonstrates a commitment to a SMC lineage. * indicates significance from the undifferentiated control represented as a normalized mean ± SD ($p < 0.05$).

Since Notch signaling has been successful in controlling phenotype switching of HCASMC, and vascular differentiation of iPSC-MSCs, 10T1/2 cell differentiation induced by Jagged1 was explored. Soluble ligands can bind to Notch receptors but are, for the most part, unable to activate signaling. Instead, they appear to block signaling induced by trans-ligands in most cases[63]. Although more commonly soluble Notch ligands have been applied to cell-based systems to competitively inhibit Notch signaling, activation through soluble Notch signaling has been highly

debated and seems to be highly context-dependent, as discussed in **Section 2.6.1.** In primary cells only cochlear[24] and human foreskin keratinocytes[22] are reported to be responsive to soluble Jagged1 to promote differentiation, supporting context dependency. Soluble Jagged1 was especially useful in differentiating MSCs into cardiomyocytes[21], peripheral blood mononuclear cells into monocytes[64], and differentiation of dendric cells[23]. This suggests the potential for soluble Jagged1 to be effective in stem cell and progenitor cell differentiation. Thus, the effect of soluble Jagged1 delivery on 10T1/2 cell response was studied. As shown in **Figure 4-17,** neither undifferentiated nor pre-differentiated 10T1/2 cells were responsive to s-Jagged1. Notch activation monitored by *HES1* was not significantly changed, and cells did not express increased smooth muscle cell markers *Acta2* and *Cnn1*.

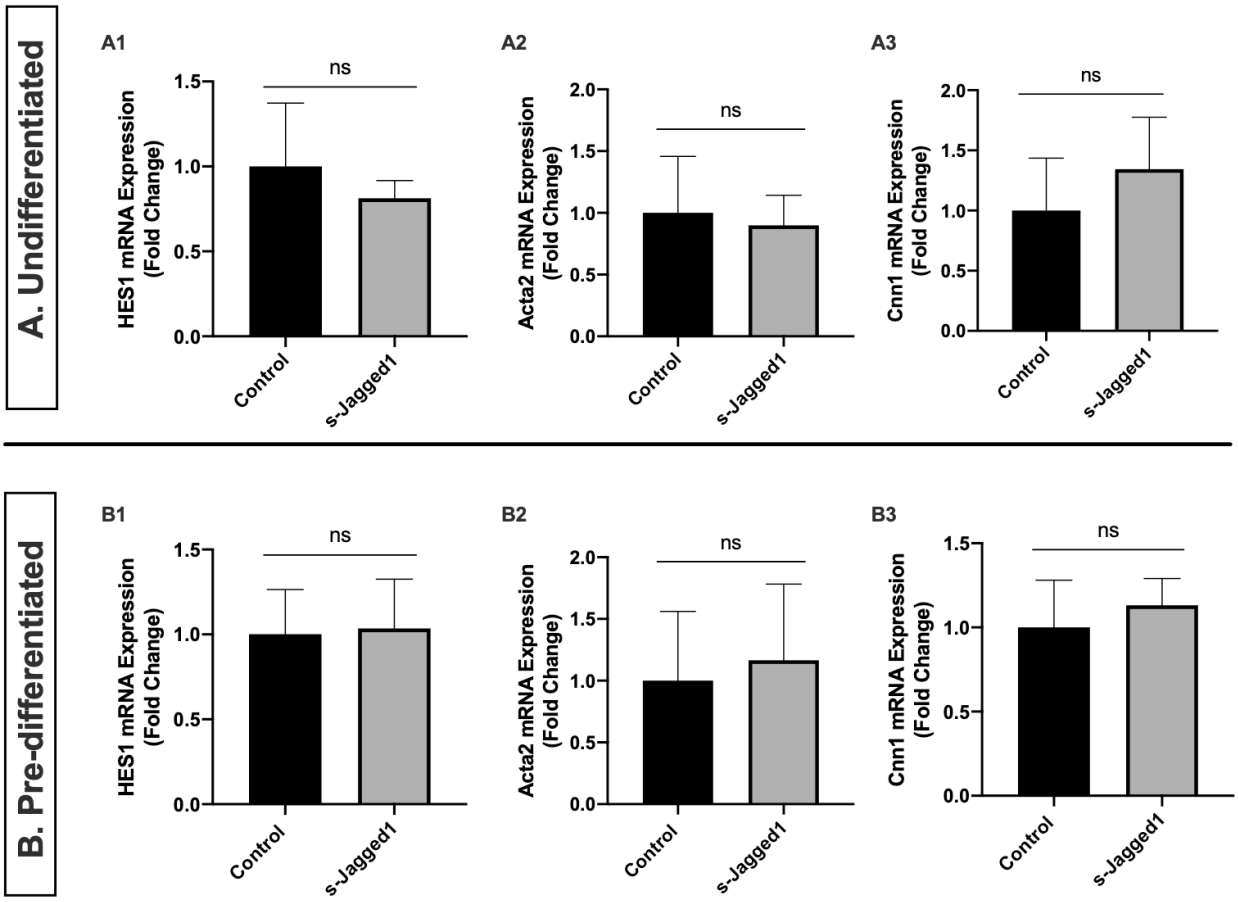


Figure 4-17 The effect of soluble Jagged1 delivery on multipotent 10T1/2 cells
10T1/2 cells were cultured for 3 days in DMEM with or without 2ng/mL TGFβ1. Both pre-differentiated and undifferentiated 10T1/2 cells were treated with soluble Jagged1 at a concentration of 2.5μg/mL for an additional 3 days. RT-qPCR analysis of *HES1*, *Acta2*, and *Cnn1* were used to show Notch specific differentiation of 10T1/2 cells. No response to s-Jagged1 indicates soluble Jagged1 was insufficient to differentiate 10T1/2 cells into a SMC lineage. The data is represented as a normalized mean ± SD. * indicates significance from the pre-differentiated or undifferentiated control, respectively at $p < 0.05$.

Previously, Jagged1-selective Notch signaling has been linked to smooth muscle cell differentiation via a RBP-Jκ-dependent pathway in 10T1/2 cells by forced Notch1-NICD expression[65]. Therefore, the influence of immobilized Jagged1 on 10T1/2 Notch3-directed SMC vascular differentiation was analyzed. Undifferentiated and pre-differentiated 10T1/2 cells were treated with Jagged1 immobilized beads according to the protocol shown in **Figure 4-18 A.**

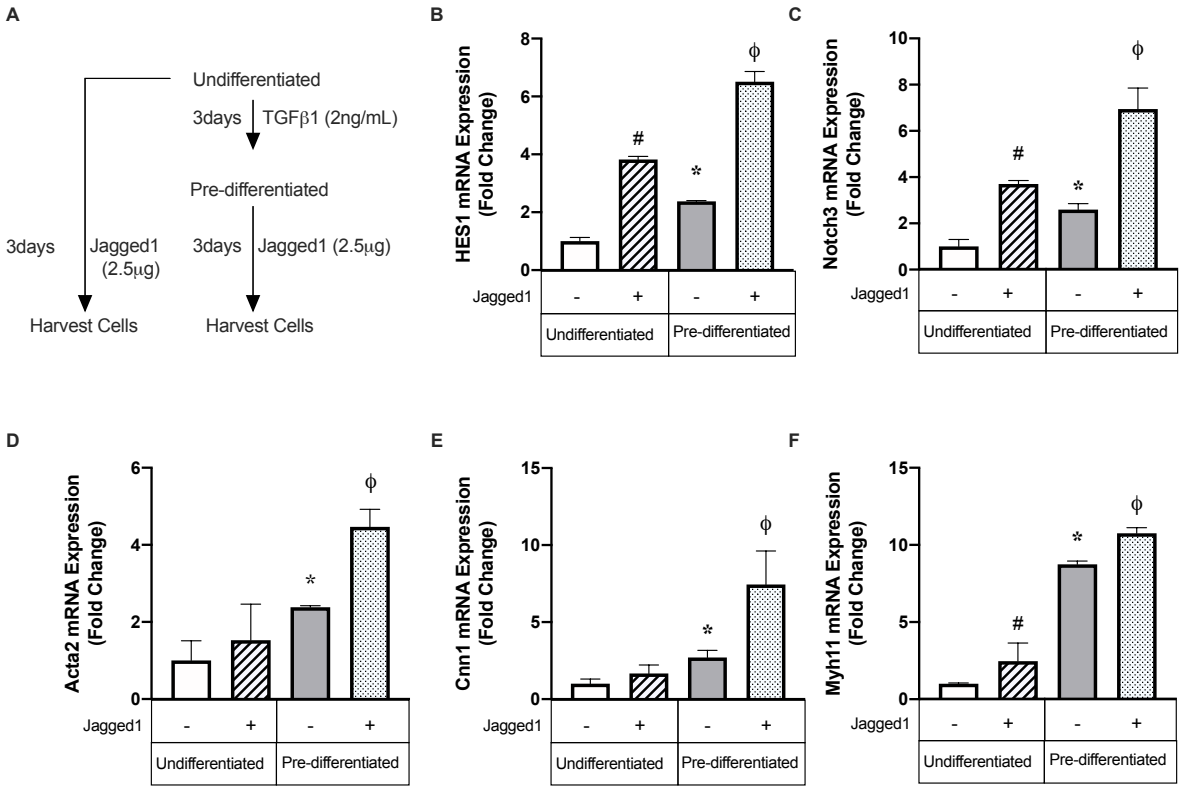


Figure 4-18 Response of pre-differentiated and undifferentiated 10T1/2 cells by Jagged1 directed Notch signaling.
10T1/2 undifferentiated and pre-differentiated (2ng/mL TGFβ1 for 3 days) were cultured for 3 days in the presence of Jagged1 immobilized beads (200 beads/cell and 2.5μg) **(A).** RT-qPCR analysis of **B)** *HES1***,** and **C)** *Notch3* as well as contractile SMC markers **D)** *Acta2,* **E)** *Cnn1*, and **F)** *Myh11* were used to show vascular differentiation. The addition of Jagged1 to undifferentiated and pre-differentiated 10T1/2 cells show that pre-differentiation improved Notch response in 10T1/2 cells. Only the upregulation of HES1 and Notch3 in undifferentiated 10T1/2 cells demonstrate a Jagged1 Notch response. The symbols indicate significance with respect to the control represented as a normalized mean ± SD ($p < 0.05$).

Although undifferentiated 10T1/2 cell expression of *HES1* and *Notch3* were responsive to immobilized Jagged1 beads, SMC contractile protein expression was not significantly upregulated (**Figure 4-18 D-F)**. 10T1/2 cells were more responsive to Jagged1 after pre-differentiation with 2ng/mL of TGFβ1. Upon pre-differentiation, a significant fold increase of *HES1*, *Notch3*, and all evaluated SMC genes were responsive to the addition of 2.5μg of Jagged1. This implies that 10T1/2 cells may need a partial commitment to a SMC lineage for Jagged1 directed Notch

signaling has a strong influence on cell response. Without pre-differentiation of these cells, it is possible that Jagged1 might direct differentiation to other cell lineages which may be a possible avenue to explore further. To ensure the Notch specific response of these genes, DAPT inhibition was used (**Figure 4-19).** Pretreatment of differentiated 10T1/2 cells with 10μM DAPT was used to inhibit S3 cleavage when treated in conjunction with Jagged1 immobilized beads, shown in **Figure 4-19 A**. All genes except for myosin heavy chain were responsive to Notch inhibition, indicating a link between regulation of these genes and Notch activation.

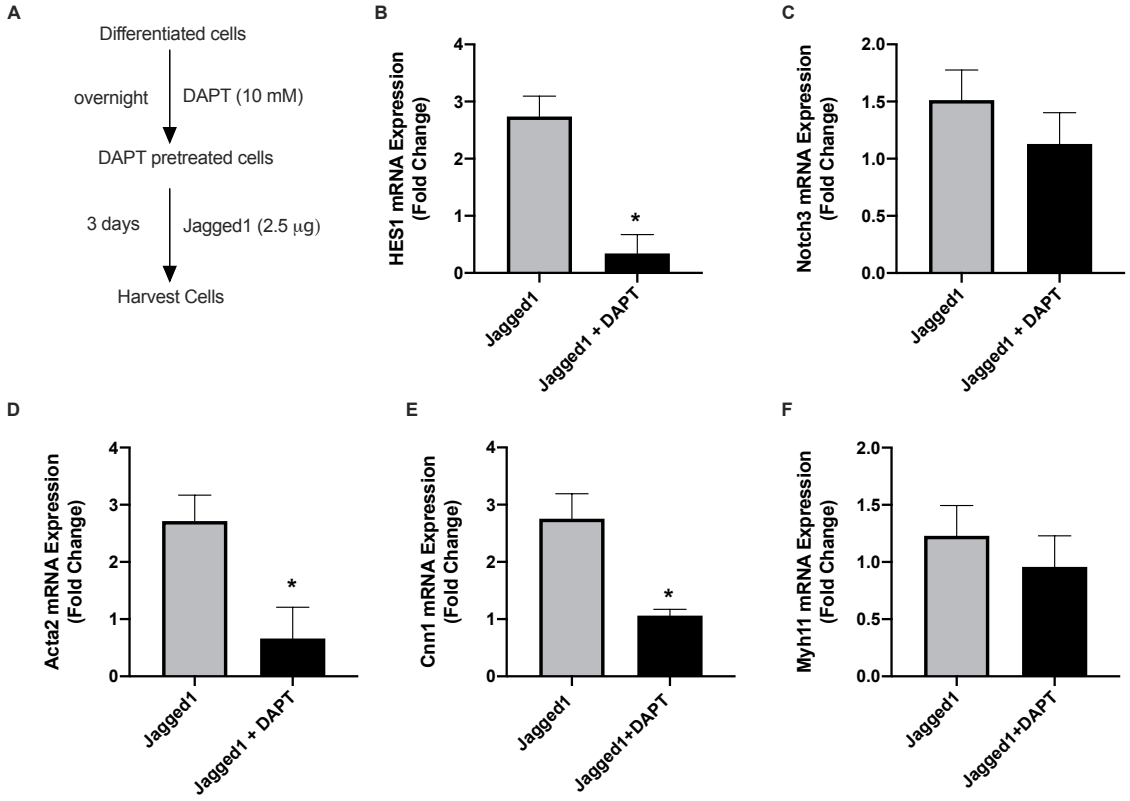


Figure 4-19 Notch specific differentiation of 10T1/2 cells
Pre-differentiated 10T1/2 (treated with 2ng/mL TGFβ1 for 3 days), were pretreated with 10 mM DAPT overnight then cultured for 3 days in the presence of Jagged1 immobilized beads (200 beads/cell and 2.5μg/mL). The scheme shown in **A)** Cell response was compared to Jagged1 treated cells alone and normalized to 10T1/2 cells cultured alone. Pre-differentiated 10T1/2 cells treated with Jagged1 with DAPT showed significant downregulation of target genes **B)** *HES1,* **C)***Acta2,* and **D)***Cnn1.* * indicates significance from Jagged1 treated 10T1/2 cells, and data are represented as a mean ± SD and normalized to the pre-differentiated control ($p < 0.05$).

Protein expression was used to confirm that these cells are being directed towards a SMC lineage. SM α-actin was observed in both immunofluorescence imaging and Western blot (**Figs. 4.20 and 4.21**). Although this data is promising, Jagged1 treatment was insufficient to show a clear increased expression of smoothelin immunofluorescent staining (not detectable), which would confirm and SMC phenotype. SM α-actin is a SMC marker but is also found expressed in other cell types; thus, it is unclear if there is a mixed cell population. Therefore, further work would be needed to explore other methods to promote a fully differentiated SMC phenotype.

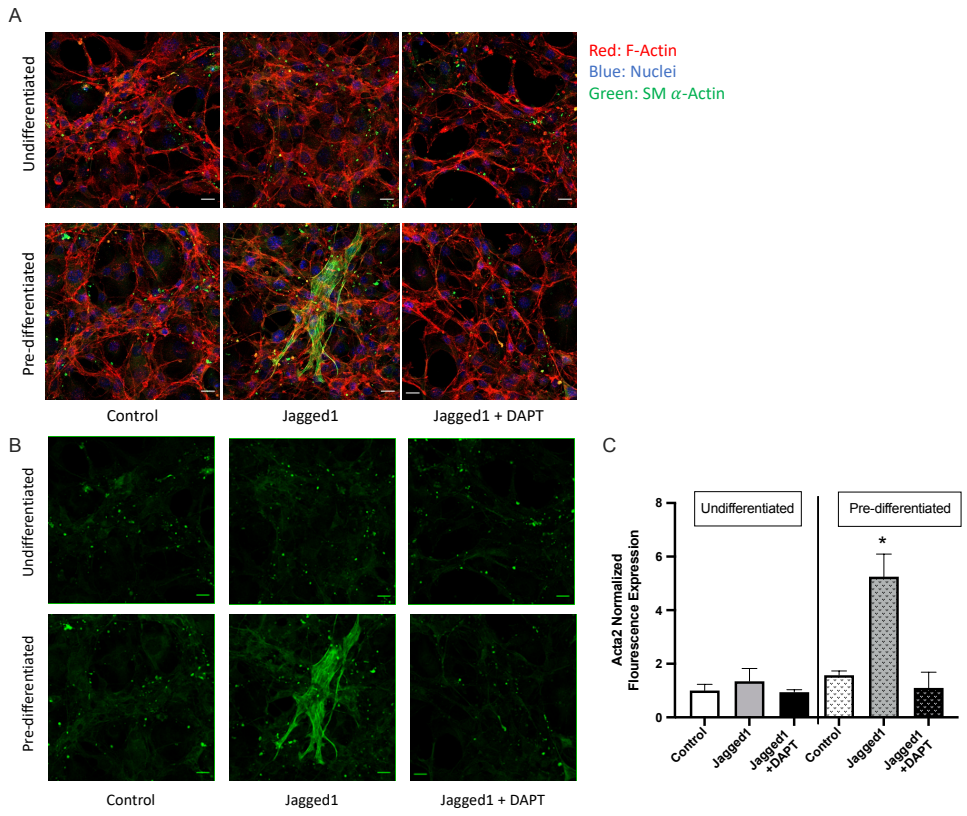


Figure 4-20. Immunofluorescence microscopy of undifferentiated and pre-differentiated 10T1/2 cells treated with Jagged1
Undifferentiated or pre-differentiated (treated with 2ng/mL TGFβ1 for 3 days) 10T1/2 cells were plated on glass cover slides at a density of 30 000 cells/ slide, and then treated with Jagged1 beads (200 beads/cell and 2.5µg/mL) or Jagged1 beads + DAPT (10mM). F-actin (red), nuclei (blue) and Acta2 (green). The corresponding quantification of the images. Scale bar = 50μm.

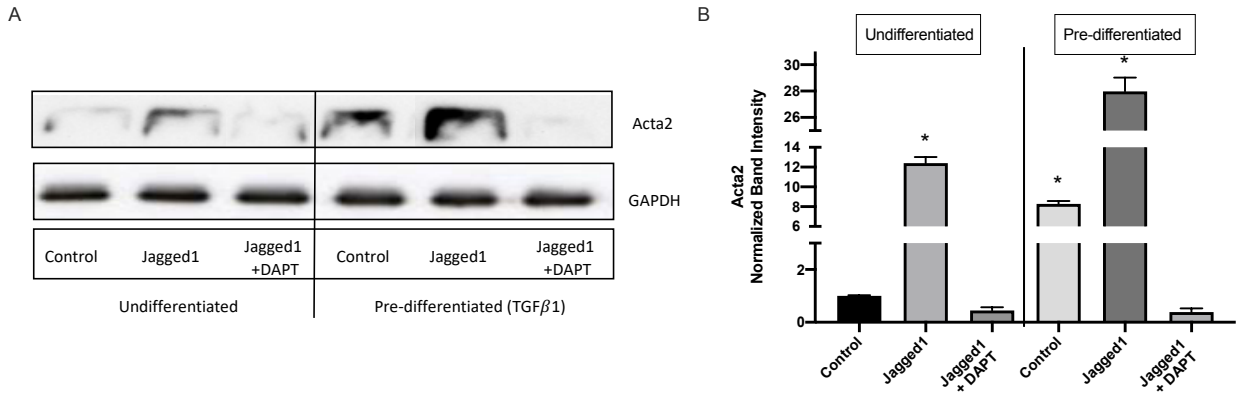


Figure 4-21 Western Blot protein analysis of undifferentiated and pre-differentiated 10T1/2 cells treated with Jagged1
Undifferentiated or pre-differentiated (treated with 2ng/mL TGFβ1 for 3 days) 10T1/2 cells were plated and then treated with Jagged1 beads (200 beads/cell and 2.5μg/mL) or Jagged1 beads + DAPT (10mM). Jagged1 beads upregulated Acta2 protein in differentiated 10T1/2 cells. Western blots are shown in **A).** The relative band intensity is quantified in the bar diagrams on the right and were normalized to the GAPDH control **B),** and relative expression expressed as a fold increase compared to the undifferentiated control. Data is represented as a mean ± SD. ($p < 0.05$)

Taken together, although the influence of immobilized Jagged1 delivery on the differentiation of these cell types into a smooth muscle cell lineage was insightful, other biochemical (e.g., ascorbic acid) or topographical (e.g., 3D scaffolds) need to be investigated to achieve a fully mature SMC for vascular tissue engineering.

4.3. Molecular mechanotransduction and Notch3 signaling

The final objective in this thesis was to explore Notch mechanotransduction to potentially enhance signaling efficacy because Notch signaling has been shown to be a mechanosensitive pathway[66,67]. The concept of a pulling force requirement for Notch activation has been around for nearly two decades; however, only recently have studies used molecular force measurements to prove this hypothesis. As discussed in **Section 2.7.2**, a tension force delivered to the receptor-ligand complex permits a conformational protein unfolding, which allows the active site for ADAM cleavage (S2) to be available. This conformation change is then able to activate

downstream targets of the Notch pathway. There are also conflicting reports about the responsibility of receptor clustering and oligomerization and their force contribution to Notch activation[66,68,69].

Mechanical loading has appeared to be sufficient to activate the receptor in absence of the native cell-bound ligand[70]. Force-dependent shedding of the NECD was also confirmed by utilizing 1:1 (bead:receptor) binding of magnetic nanoparticles to activate single Notch receptors. Therefore, it is believed that a mechanical force, in addition to ligand-receptor binding, is needed for Notch activation and demonstrated with DLL1[11,71], and DLL4 [70] ligands. Since there are structural differences between Notch ligands, it is not known if mechanical loading of Jagged1 is needed to activate Notch signaling in SMCs. It is postulated that additional biochemical or structural cues that are not offered by the Jagged1-immobilized beads may be necessary to activate the signaling cascade that results in SMC Notch activation, but this is unknown. In view of the above discussion, the next objective is to evaluate whether or not the Jagged1-Notch3 pairing is mechanosensitive.

4.3.1. The effect of tension force for Notch3 activation and phenotype control of smooth muscle cells

Jagged1-conjugated magnetic nanoparticles were used as a tool to study how applying tension to cells can change the activation of Notch signaling using a magnetic tweezer setup. The magnetic tweezer set up was selected when compared to other force application techniques because the versatility of attachment to the beads, low heat/photodamage as seen with optical tweezers, the ability to achieve a constant force, and the ability of the tweezer setup to apply large force ranges. Although this platform is limited because of the magnetic variability in the bead population, the

low resolution if using a video-based bead detection system, and lack of knowledge on eliminating the torque on the beads, this system was acceptable for the present study (**Figure 4-22**).

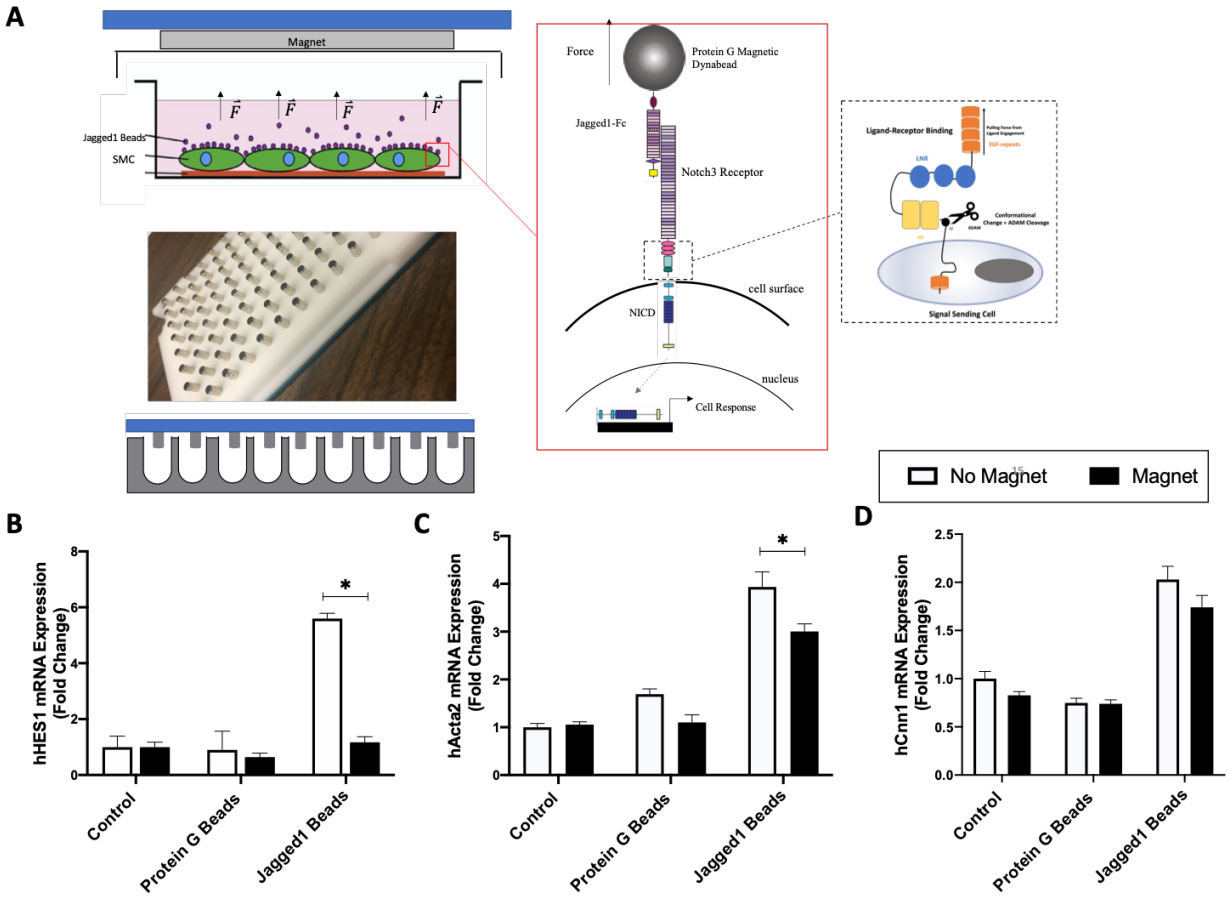


Figure 4-22 Mechanosensitivity of HCASMC to Jagged1 under tension
HCASMC were cultured in a 96-well plate for 2 days to allow for cell spreading and attachment, then treated with Jagged1-immobilized beads. Cells were cultured for another 2 days under the application of a magnetic tension force. Expression levels of *HES1*, *Acta2*, and *Cnn1* were quantified with RT-qPCR and normalized to the control cultures without the magnetic plate. The bead concentration was 200 beads/cell and 2.5µg/mL of Jagged1 protein was immobilized to that bead concentration. Data are represented as mean ± SD. * represents significance at $p < 0.05$.

When cells expressing the Notch3 receptor were incubated with Jagged1-immobilized magnetic nanoparticles, there was a significant increase in contractile gene expression upon addition of the Jagged1 beads demonstrating that our system works in a 96-well plate format. Upon

application of a magnetic tension force to HCASMCs there was no significant cellular response, demonstrating that the force magnitude or the magnetic beads alone are not affecting the signal. Upon treatment with Jagged1 beads and a magnetic tension force, a significant downregulation of *HES1* and *Acta2* but not *Cnn1* was observed in comparison to the Jagged1 treated cells with no magnet. These results were confirmed in 10T1/2 cells. Pre-differentiated 10T1/2 cells were used because they were proven earlier to be more responsive to Jagged1. Similar to what was seen in the HCASMCs, the addition of a magnetic plate to Jagged1 treated cells downregulated *HES1* and *Acta2* gene expression (**Figure 4-23)**. The magnetic tension force had no effect on the cells cultured without beads.

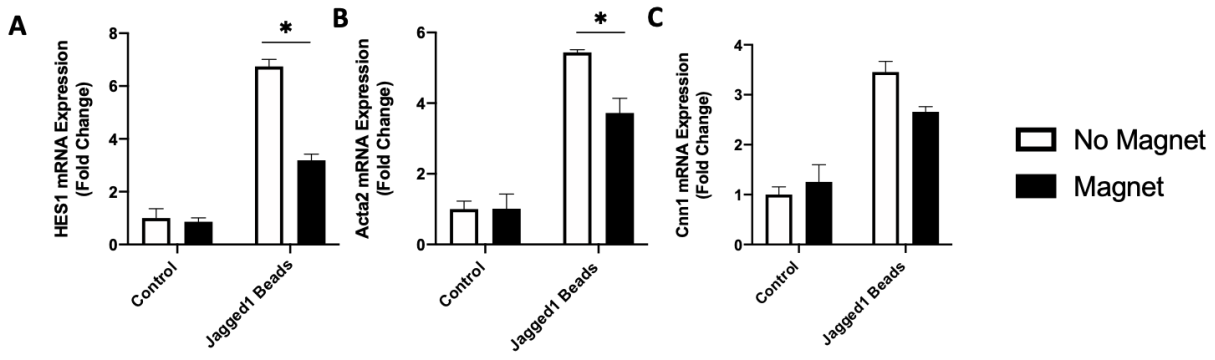


Figure 4-23 Mechanosensitivity of 10T1/2 cells to Jagged1 under tension
Pre-differentiated 10T1/2 cells were cultured in a 96-well plate for 2 days to allow for cell spreading and attachment, then treated with Jagged1-immobilized beads. Cells were cultured for another 2 days under the application of a magnetic tension force. Expression levels of *HES1*, *Acta2*, and *Cnn1* were quantified with RT-qPCR and normalized to the control of pre-differentiated 10T1/2 cells cultured alone with no magnetic plate. The bead concentration was 200 beads/cell and 2.5μg/mL of Jagged1 protein was immobilized to that bead concentration. Data are represented as mean ± SD. * represents significance at $p < 0.05$.

By controlling the distance between the cells and the magnet, it is possible to vary the force applied to the cells as a function of the height from the PDMS surface to the plate lid. To present cells at various heights, PDMS polymer was dispensed into the culture chamber, creating a

"terraced" configuration with wells of different depths across the plate.[70] Various heights were tested by adding different PDMS, including the addition of the reported 100μL corresponding to ~2pN force. As shown for both HCASMC and 10T1/2 cells, increasing the height did not significantly affect *HES1* gene expression and further downregulated *Acta2* (**Figure 4-24 A, B)**. Similar results were found for other heights with PDMS volumes ranging from 0-150μL (~ 0.5-5 pN), (data not shown). Suggested by this data, and contrary to previous reports that force application would improve signaling efficacy[72], the application of a magnetic tension force as a function of height downregulated expression of *HES1* and contractile marker *Acta2*. It appears that the mechanosensitivity of Notch ligand-receptor pairing is context-dependent, similar to the varying responses that are seen with activation or inhibition of the various ligand-receptor combinations. Although Delta-like ligands have been shown to benefit from a molecular force, this may not be the case with the Jagged1, specifically the Jagged1-Notch3 pairing in the SMC. It suggests that the bead-bound Jagged1 provides enough traction force for Notch activation.

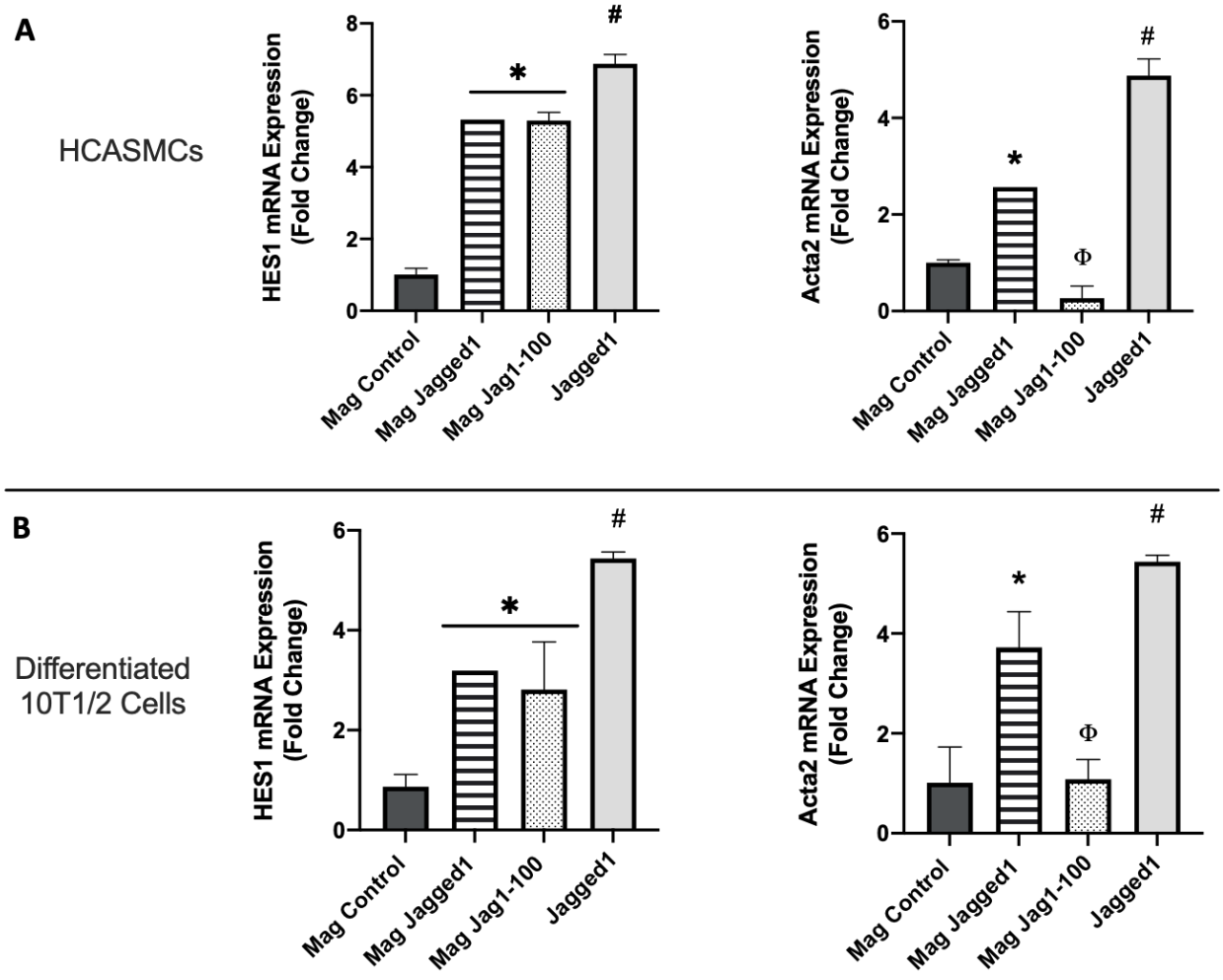


Figure 4-24 Height and sensitivity on smooth muscle cell response
To create a terraced configuration of the 96-well plate, 100μL of PDMS was dispersed into the well to increase the force magnitude applied to the cells. Force magnitude is a function of PDMS height. The PDMS surface was then sterilized and treated with fibronectin. HCASMC or differentiated 10T1/2 cells were plated on the two different well heights in the 96 well plate for 2 days to allow for cell spreading and attachment, then treated with Jagged1-immobilized beads. Cells were cultured for another 2 days upon application of a magnetic tension force. Expression levels of *HES1* and *Acta2* in both HCASMCs and pre-differentiated 10T1/2 cells were quantified with RT-qPCR and normalized to the control of cells cultured alone with the addition of a magnet. The bead concentration was 200 beads/cell and 2.5μg/mL of Jagged1 protein was immobilized to that bead concentration. Data is represented as mean ± SD. Symbols indicate significance from each other ($p < 0.05$).

The finding that Jagged1-Notch3 pairing to be unresponsive to stretch forces provide insight from a physiological perspective. In the native vasculature, endothelial cells are mechanosensitive and are influenced by shear stress driven by blood flow in the vessel wall. The VSMCs in the vascular wall experience strain due to the distention force and higher strain is found in thinner vessels than in thicker vessels. The Jagged1-Notch pairing has been shown to be mechanosensitive and influenced by strain and blood pressure in the vascular wall[73,74]. VSMCs to 10% uniaxial strain demonstrated reduced expression of *Notch3, Jagged1, HEY1, HEY2, and HES1*, and decreased further with increasing strain[75]. Furthermore, when looking at an array of receptors, ligands, and transcription factors, Notch3 was the only receptor with a strain-responsive decrease in expression, unlike Notch1 and Notch2. As for the ligands, Dll1 increased with increased strain, and Jagged1 decreased with increasing strain. *HES1, HEY1,* and *HEY2* also decreased with increasing strain. Together, this demonstrates that while Dll1 ligand is positively influenced by strain Jagged1 in comparison is negatively affected, these cited studies, along with the present data, indicate that Jagged1 may be negatively regulated by strain.

Additionally, vimentin networks have also been shown to effectively disperse locally induced mechanical stress into larger regions enabling the dissipation throughout the cell[76]. Importantly vimentin has also been linked as a requirement for efficient receptor-ligand transendocytosis[77]. Vimentin has been reported as an important protein for Notch transactivation by ECs, and proximity ligation assays have demonstrated direct vimentin interaction with Jagged1[77]. Vimentin knock-out mice showed disruption of VSMC differentiation and adverse remodeling[75]. Without vimentin, there is a reduced structural component in the cytoskeleton, which may be responsible for endocytosis and the pulling force to create NECD unfolding. However, Jagged1 beads were able to rescue the maturation of SMCs in vimentin knock-out mice, which may indicate that no

pulling force caused by trans endocytosis may be required for Notch activation. Therefore, and similar to variable differentiation and cell response by transactivation Jagged1 vs delta-like binding, there may be differences in the mechanosensitive nature of the ligands.

Lastly, as suggested earlier, clustering and oligomerization may also support the pulling force for Notch activation. Thus, when Jagged1 is attached to Protein G beads via affinity immobilization, the Jagged1-Fc fusion proteins may form pre clustered dimmers owing to the Fc domain. While these clusters may not be capable of forming polymeric aggregates to generate limited traction for the pulling force, it may be sufficient for Notch activation in SMCs, and thus any additional strain to the surface would mimic increased blood flood in the vessel and negatively affect Jagged1 signaling. More investigation is needed to elucidate this.

A schematic for translating the current approach into an automated magnetic tweezer set-up is shown in **Appendix A4.** This design was inspired by other magnetic tweezers designs[78–81], and would be useful to achieve greater force ranges, improved accuracy, and improved visualization of cell and bead behavior in real-time, which are all limitations of the current study.

4.4. References

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Chapter 5. Conclusions and Future Directions

5.1. Conclusions

Notch signaling has been identified as a promising target for many cellular therapies and shows considerable progress and potential in directing and reprogramming cellular behavior. Notch signaling can be utilized to engineer pre-vascularized thick tissues or to develop anti-angiogenic cancer therapeutic strategies. Depending on the context, activating or suppressing Notch signaling is a valuable tool to engineer tissues for therapeutic or diagnostic use. This, in turn, allows creating diseased model tissues for studying drug discovery and screening. It has already been demonstrated as a potential strategy to prevent atherosclerosis[1], promote cardiac valve regeneration[2], reduce cell senescence in cell sheet engineering[3], and to engineer vascular tissues[4,5]. Specifically, Jagged1-Notch3 communication has been proven important in artery homeostasis, and Jagged1 has been suggested as a promising target to maintain SMCs in a contractile phenotype following injury. There has been considerable progress already made in the development of Notch signaling biomaterials. While immobilized Jagged1 has been proven as a promising biomolecule to control SMC response, an ongoing challenge is developing proper biomaterial delivery platforms and obtaining mature expression of contractile proteins. This thesis works towards enhancing our knowledge of Jagged1 delivery in SMC phenotype control and differentiation in the vasculature.

The data in this thesis presented was able to regulate phenotype switching of HCASMCs using bead-bound Jagged1, by inducing HES1 a Notch transcription factor, and early-stage contractile markers, SM-actin, and calponin. To induce late-stage contractile marker co-delivery and cross talk between Jagged1 and TGFβ1 was necessary. To translate these findings to other vascular smooth muscle cell sources, bead-bound Jagged1 was investigated to direct iPSC-MSC and 10T1/2

cells towards a vascular lineage commitment. Although Jagged1 successfully directed the commitment of these cells towards a SMC lineage, these cells lacked mature contractile markers, including myosin heavy chain and smoothelin. Pre-differentiation of 10T1/2 cells was also needed for increased Jagged1 response. Thus, it was concluded that Jagged1 should be studied in combination with other factors to induce a fully differentiated phenotype. Lastly, this thesis demonstrated that bead-bound Jagged1 did not require an additional tension force for Notch activation which has been needed for Delta-like ligands.

Designing signal-presenting biomaterials for directed cellular therapies is a complex process that requires precise control of signals. In conclusion, this research suggests promising therapeutic potential for the Jagged1 ligand. In the long-term, these results could potentially be used for Jagged1 stent technology to control vessel homeostasis until regeneration can occur following stent deployments.

5.2. Future directions

Many researchers have largely focused on the cellular response-driven through a biochemical perspective, but a major limitation in engineered tissues is driven from a biomaterial perspective. Designing cell-instructive biomaterials incorporating the biological activity of proteins is an emerging field. These biomaterials could be applied to the Notch signaling system to create a more dynamic microenvironment and introduce tissue mechanics and activate mechanosensitive receptors with various forces introduced. Since endocytosis of the ligand following receptor binding generates the force to render Notch S2 cleavage, it implies that attaching the ligand to non-dynamic surfaces may not be sufficient to activate Notch. Three strategies to address this could be by use of supramolecular biomaterials/scaffolds that having dynamic, interchangeable, and reversible motifs [6,7], utilizing chemical spacers to provide more ligand-receptor dynamic

interacted, or by reverse engineering of the Notch receptor for reduced pulling force dependency[8]. Since the latter strategy involves transfection for synthetic Notch expression, supramolecular materials or biomaterials incorporating biochemical spacers may be an attractive avenue from a tissue engineering perspective. Regardless of the strategy to activate Notch signaling, it remains to be an excellent tool for tissue engineering and regenerative medicine.

Future research would include dynamic interfaces at the biomaterial surface by introducing chemical spacers. Research suggests that optimal ligand surface coverage can be maximized with spacers due to the ability of the polymer-bound proteins to form a thick layer and dispersing the ligands in space to optimize binding and minimize lateral repulsions[9]. Direct attachment of a protein to a surface without a spacer can cause steric hindrance and reduced bioactivity of the immobilized protein. In addition, without a spacer, multiple contacts between protein and nanoparticle surface are more probable favoring total or partial protein denaturation and thus decreasing protein activity[10]. There is some evidence for target selectivity of specific Notch receptors by which the ability of Notch ICDs to form dimers might influence the activation of downstream targets, including receptor recruiting of gene response[11]. Interestingly, the configuration of CSL binding sites appearing as monomer or dimers have influenced the likelihood of recruiting Notch1 or Notch3 ICD, respectively[12]. To mimic the dynamic regulation of signaling ligands, polymer chemistry can be harnessed to create chemical spacers to improve biomolecular recognition, ligand accessibility, and dynamic behavior of immobilization[13].

Once the optimal strategy for presenting Jagged1 on a material surface is determined, immobilization of Jagged1 in a 3D microenvironment or to stents would be the next step to developing a functional treatment. As proposed, Notch functionalized vascular stents would be an effective treatment to maintain vessel homeostasis and control SMCs until vessel regeneration can

occur post angioplasty surgery. To test Jagged1-functionalized vascular stents, the response of SMCs in a tissue-engineered vascular model to determine the effectiveness of this proposed treatment is needed.

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Appendix A1: Advantages and disadvantages of current stent technology

Stent Type	Composition	Advantages	Disadvantages
a) Bare metal stents	**Stents** • 316L stainless steel • platinum-iridium alloy • tantalum, nitinol, cobalt-chromium alloys • titanium	• Maintains open artery and reduces vessel closure * • Provides structural support * • Regains proper blood flow * • Less invasive than bypass grafts*	• Metal scars endothelial tissue • Inert- no bioactivity • Prevents elastic recoil and remodeling • Neointimal growth response • High rates of restenosis • Nondegradable
b) Stent coatings	**Stent +Coating** • Gold, silicon-carbide • Iridium oxide • Diamond-like carbon	• Reduced surface energy • Smoother surface textures • Neutralized/stabilized surfaces • Enhanced oxide layer • Reduces metal leaching	• No bioactivity • High rates of restenosis • Nondegradable
c) Drug eluting stents	**Stent + Coating + Drug** • Paclitaxel, • Everolimus, • Sirolimus, • Myolimus • Novolimus	• Drugs mitigate adverse response to metal • Drugs target immunorejection, proliferation, and antithrombosis • Reduces early restenosis*	• Incomplete healing • Includes chronic inflammatory response • Increased risk of late stent thrombosis • Need for antiplatelet therapy • Nondegradable
d) Bioresorbable stents	• metallic (iron-based or magnesium-based) alloys • Polymers ex. PLLA and PDLLA	• Expansive remodeling possible • Degradable • No need for prolonged double antiplatelet therapy • Restoration of vasomotion • Biocompatibility	• Not permanent-the artery must regenerate at equal time of stent degradation • Thicker and wider struts to gain mechanical integrity • Larger catheter profile
e) Bioactive stents	• heparin[1] • tripeptide Arg-Gly-Asp [2,3] • vascular endothelial growth factor (VEGF) • small interfering ribonucleic acid (siRNA) nanoplexes	• Potential recovery of biological function • Controlled targeted delivery • Enhance re-reendothelialization • Bioactivity • Good biocompatibility	• Nondegradable • Late-stage stent thrombosis • In-stent restenosis

***indicates that this characteristic is common for all thereafter stent types** **References: [4–9]**

Appendix A2: Jagged1 biomaterial immobilization

Immobilized Jagged1 for bone tissue engineering

Authors	Year	Journal	Cell Type	Application	HES1	HEY1	ALP	COL1	BSP	OSX	TWIST2	Other	Ref.
Zhu et al	2013	Stem Cells	MSCs	osteogenic differentiation	↑	↑	↑		↑	↑			[10]
Dishowitz et al.	2013	Journal Biomedical Material Research Part A	bone marrow (BM) MSCs	fracture repair		↑	↑						[11]
Sukarwan et al	2016	Archives of Oral Biology	Stem cells in human exfoliated deciduous teeth	osteogenic differentiation	↑	↑	↑	↑			↓	(NS) OPN, (NS) OCN	[12]
Manokawinchoke et al.	2017	Scientific Reports	dental pulp MSCs	odonto differentiation	↑	↑	↑	↑		↑	↓	↑ BMP2, ↑RUNX2	[13]
Ndong et al.	2018	Journal Biomedical Material Research Part A	fibroblast-like cells from embryonic palatal shelves	osteogenic differentiation	↑	↑	↑	↑	↑			↑ RUNX2	[14]
Nowwarote et al	2018	Archives of Oral Biology	periodontal ligament cells	osteogenic differentiation	↑	↑	↑						[15]
Osathanon et al	2019	Archives of Oral Biology	alveolar and iliac BM-MSCs	promote bone mineralization	↑	↑	↑	↑			↓		[16]

Immobilized Jagged1 for other applications

Source	Year	Journal	Tissue	Cell Type	Application	Notch Markers	Markers	Ref.
Beckstead et al, 2006	2006	Journal of Biomedical Materials Research	Epithelial	Rat esophageal epithelial cells	Epithelial differentiation and stratification	↑ CBFJ-luciferase	↑Caspase3, ↑Occludin ↑involucrin, ↑	[17]
Gancalves et al, 2009	2009	Biomaterials	Blood	HL-60 leukemia cell line	blood cell expansion	↑HES1	N/A	[18]
Osathanon et al, 2013	2013	Stem Cells and Development	Neurogenic	periodontal ligament derived MSCs	Neurogenic commitment	↑HES1, ↑HEY1	↑SOX2 ↑B3-tubulin	[19]
Tung et al, 2014	2014	Stem Cell Reports	Cardiac	embryonic Stem cells	Cardiac and ectodermal differentiation	↑ HES1	↑SOX1 ↑cardiac troponin T ↓SM-Actin	[20]
Boopathy et al, 2014	2014	Biomaterials	Cardiac	cardiac progenitor cells	differentiation into cardiac lineage	↑HEY 1	↑nkx2-5, ↑MEF2C, ↑GATA4	[21]
Wen et al, 2014	2014	Applied Materials and Interfaces	Cardiac	BM-MSCs	myocardial lineage commitment	↑HES1, ↑Notch1	↑cTnt(Troponin) ↑Nkx2.5 ↑MYH7,↑GATA4	[22]
Ji et al, 2016	2016	Cellular Physiology and Biochemistry	Pericytes	Hemangioma pericytes	Pericyte Phenotype	↑HES1, ↑HEYL, ↑Notch3	↑SM-MHC, ↑SM actin, ↑myocardin	[23]
Izadi et al, 2018	2018	Biomaterials	Immunoprotection	pancreatic islets	improved immunoprotection of islets	N/A	↑IL-10. ↑TGF-beta, ↓IFN-gamma, ↓TNF-alpha	[24]
Negri et al, 2019	2019	Scientific Reports	Epidermal	epidermal keratinocytes	Epidermal homeostasis	↑HES1, ↑IRF6, ↑Notch3	↑TGM1, ↑IVL	[25]

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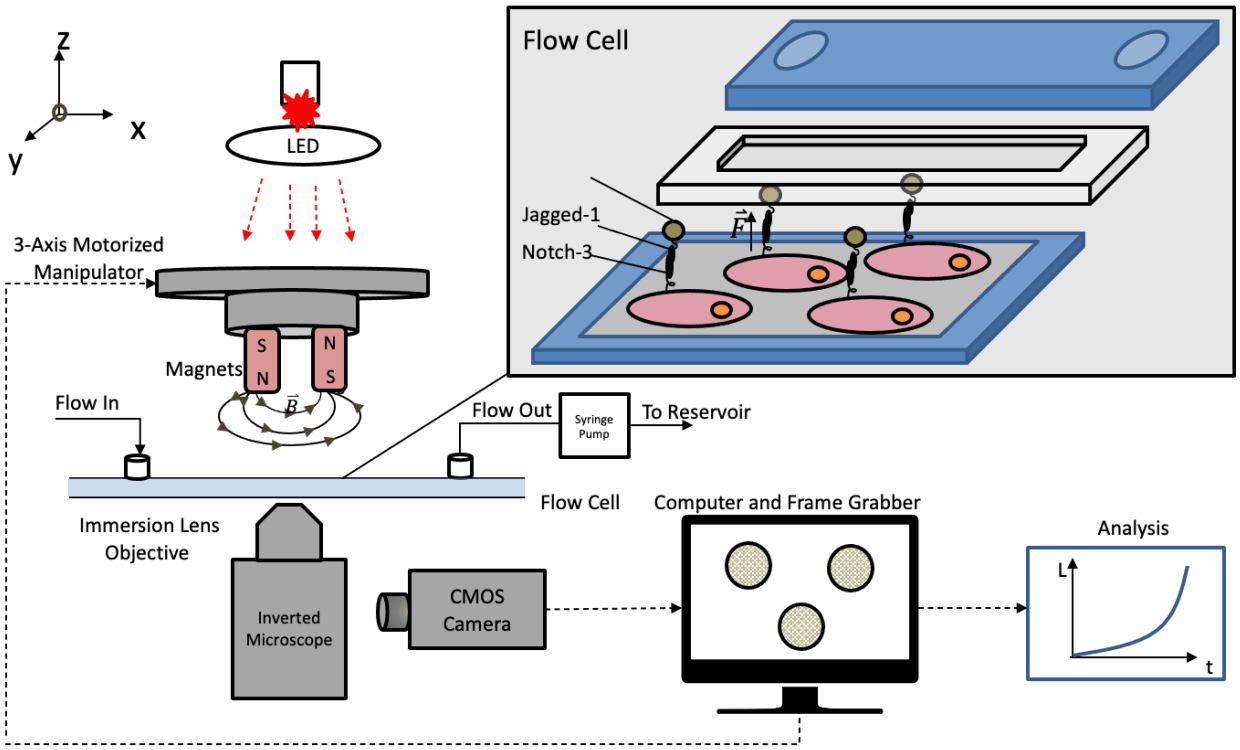
Appendix A4: Magnetic tweezer design schematic



This is a design schematic for translating the magnetic tweezer apparatus used to determine molecular mechanotransduction into an automated, microscope-integrated system. This system would allow for precise control of force application at a greater range than the permeant magnetic plate used in this thesis and a flow system to minimize contamination and achieve proper nutrient and oxygen delivery to cell cultures. Integrated into the system is also a camera for imaging cellular response and bead behavior in real-time. Although the Jagged1-Notch3 system shows a decreased expression of contractile marker expression upon force application, this system could be used for force application in other cell systems.

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Conference Oral Presentation

Jagged1 Presenting Cell Surrogate Biomaterials

Tissue Engineering and Regenerative Medicine International Society (TERMIS) Americas Chapter Meeting

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December 2-5, 2019

Review Paper

Kathleen Zohorsky, Kibret Mequanint. **Designing biomaterials to modulate Notch signaling in tissue engineering and regenerative medicine.** *Tissue Eng. (B).* **2020** doi.org/10.1089/ten.TEB.2020.0182

Research Paper (under preparation)

Kathleen Zohorsky, Shigang Lin, and Kibret Mequanint. **Surface-bound Jagged1 induces differentiation and phenotype control of vascular smooth muscle cells via Notch3 signaling**. (2021)